I am very proud to be called a pig. It stands for pride, integrity and guts.

- Ronald Reagan

Promoter

Prof. dr. ir. Stefaan De Smet
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Melle, Belgium

Copromoters

Dr. ir. Joris Michiels
Department of Applied Bioscience, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Prof. dr. Chris Van Ginneken
Department of Veterinary Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Antwerp, Belgium

Members of the examination committee

Prof. dr. ir. Mieke Uyttendaele (Chairman)

Department of Food Safety and Food Quality, Ghent University, Ghent, Belgium

Ass. Prof. dr. ir. Walter Gerrits

Animal Nutrition Group, Wageningen University, Wageningen, The Netherlands

Prof. dr. ir. Katleen Raes

Department of Industrial Biological Sciences, Ghent University, Kortrijk, Belgium

Prof. dr. Richard Ducatelle

Department of Pathology, Bacteriology and Poultry diseases, Ghent University, Merelbeke, Belgium

Ass. Prof. dr. Lianqiang Che

Animal Nutrition Institute, Sichuan Agricultural University, Ya'an, China

Dean

Prof. dr. ir. Marc Van Meirvenne

Faulty of Bioscience Engineering, Ghent University, Ghent, Belgium

Rector

Prof. dr. Anne De Paepe

Ghent University, Ghent, Belgium

WEI WANG

REDOX STATUS AND DEVELOPMENT OF THE SMALL INTESTINE IN INTRAUTERINE GROWTH RESTRICTED AND NORMAL BIRTH WEIGHT PIGLETS

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

2016

Dutch translation of the title:

REDOXSTATUS EN ONTWIKKELING VAN DE DUNNE DRAM IN BIGGEN MET INTRA-UTERIEN GROEIVERTRAAGDE EN NORMAAL GEBOORTEGEWICHT

This work was funded by a PhD scholarship from China Scholarship Council (2011691004), by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) via the research project entitled 'Optimalisatie van de redoxstatus voor een betere darmgezondheid in intra-uterien groeivertraagde biggen (IWT_LO100856, 2011-2015)' and by Special Research Fund - Cofunding for Chinese candidates holding a CSC-grant (BOF-01SC1034).

To refer to this thesis:

Wang. W. (2016). Redox status and development of the small intestine in intrauterine growth restricted and normal birth weight piglets. PhD thesis, Ghent University, Belgium, 151p.

ISBN 978-90-5989-899-8

CONTENTS

LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AP-1	Activator protein-1
BCA	Bicinchoninic acid assay
BPDS	Bathophenanthrolinedisulfonic acid disodium salt hydrate
BW	Body weight
CAT	Catalase
cDNA	Complementary DNA
CFX	Cephalexin
DNA	Deoxyribonucleic acid
DNFB	Dinitrofluorobenzene
DPN	Days postnatal
DPW	Days postweaning
EDTA	Ethylene-diamineteraacetic acid
FABP	Fatty acid binding protein
FD4	Fluorescein isothiocyanate-dextran 4
FITC	Fluorescein isothiocyanate
GCL	Glutamate–cysteine ligase
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cysteine ligase, modifier subunit
gDNA	Genomic DNA
GIT	Gastrointestinal tract
GPX	Glutathione peroxidase
GSH	Glutathione
GSR	Glutathione-disulfide reductase
GSS	Glutathione synthetase
GSSG	Glutathione disulfide
GSTA4	Glutathione *S*-transferase alhpa 4
HO	Heme oxygenase

HPLC	High performance liquid chromtography
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFNβ	Interferon-β
Ig	Immunoglobulin
IL-1β	Interleukin 1β
IUGR	Intrauterine growth restriction
JAM	Junctional adhesion molecule
mAb	Monoclonal antibody
MDA	Malondialdehyde
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acids
mRNA	Messenger RNA
NBW	Normal birth weight
NF-κB	Nuclear factor kappa B
Nrf2	NF-E2 related factor-2
OF	Oxidized fat
Papp	Apparent permeation coefficient
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with TWEEN 20
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PPARγ	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real-Time quantitative reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulfatepolyacrylamide gel
SEM	Standard error of mean
SI	Small Intestine
SOD	Superoxide dismutase

TBARS	Thiobarbituric acid reactive substances
TEER	Transepithelial electrical resistance
TJ	Tight junction
TNF	Tumor necrosis factor
Trx	Thioredoxin
TXNRD	Thioredoxin reductase
V/C ratio	Villus/crypt ratio
VNN1	Vanin-1
ZO	Zonula Occluden

Chapter I

General Introduction

1 OUTLINE OF THE PHD STUDY

In the studies presented in this thesis, the overall objective is to document the developmental pattern of intestinal physiological functions with particular emphasis on barrier function and redox status in intrauterine growth restriction (IUGR) piglets, as compared to normal birth weight (NBW) ones (**Fig. I-1**). This will provide information for the nutrition and management intervention that can be offered to IUGR offspring. The specific objectives of this Ph.D. research are:

1) To evaluate whether the shift from parental to enteral nutrition at birth will induce an oxidative challenge in piglets and whether it is affected by IUGR (**Chapter II**);
2) To evaluate whether weaning will cause long-term effects in the intestinal oxidative status of piglets on different key time points postweaning, and whether this is affected by IUGR (**Chapter III**);
3) To establish an oxidative stress-inducing model and evaluate the specific critical redox couple like glutathione/glutathione disulfide, and whether it is affected by IUGR (**Chapter IV**).

In the following parts of **Chapter I,** a literature review is presented, which focus on the knowledge related with the topics in this study. This includes, 1) small intestinal mucosal physiology, barrier function and their developmental characteristics during the early life of piglets; 2) the effect of weaning on the small intestinal structure and function; 3) definition of IUGR and its effect on the small intestinal function; and 4) oxidative stress and intestinal antioxidant system.

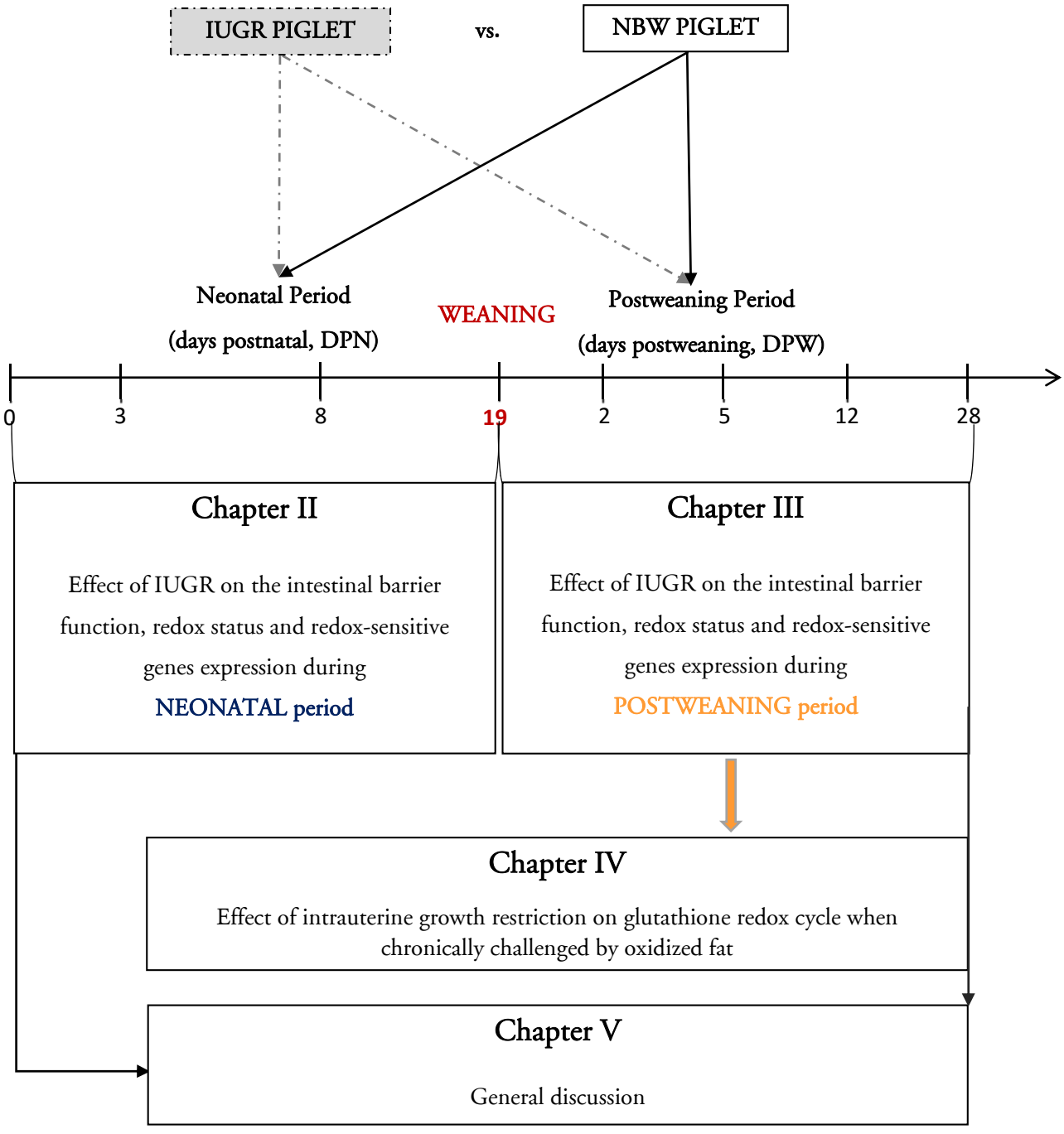

Figure I–1 *Outline of the thesis.*

2 SMALL INTESTINAL MUCOSA

2.1 Small intestinal mucosa architecture

The small intestine extends from the pylorus to the ileo-cecal valve, with three main segments, duodenum being closest to the stomach, followed by the jejunum and finally the ileum. The intestinal mucosa is a mucous membrane lining the inner part of the digestive wall. It consists of three sublayers including a single cell layer of epithelial tissue, the lamina propria mucosae, and the lamina muscularis mucosae (**Figure I–2**).

Importantly, the small intestine mucosae are characterized by finger-like projections known as villi, which extend into the lumen and increase the intestinal surface area. The surface membrane of the absorptive epithelial cells is characterized by microscopically small extensions known as microvilli. This gives the epithelial surface a brush border format. Different regions of the intestine have distinct physiological functions. The villi become progressively shorter and broader going down the length of the small intestine, which is consistent with the lower rates of digestion and absorption that occur in these regions (Mowat & Agace, 2014). Each villus is equipped with circulatory vessels. Capillaries move blood into the entire length of the villus. When the villi absorb nutrients like water soluble vitamins, amino acids and sugars, they are transported into the capillaries for distribution and use throughout the body. The villi also contain lacteals, which are part of the lymphatic system. Fat, fat soluble vitamins and excess liquids absorbed by the villi are first moved into lymph fluid circulation before being entering the blood stream. In addition, brush border enzymes secreted into the micro-villi-covered cells are capable of breaking down luminal saccharides, proteins and nucleic acids directly on the surface of their surface. When the cells on the upper surface of the villi sloughed off, these enzymes are released directly into the intestinal lumen, aiding in digestion.

Crypt is the small invagination of epithelium that separates the villi. Cells grow and divide rapidly in crypt. After differentiation, cells are arising to the top of the adjacent villi to replace cells that are

sloughed off during the movement of digesta through the intestine. The multipotent stem cells in crypts give rise to several different types of mature epithelial cells, mainly absorptive enterocytes, but also Paneth cells, goblet cells and neuroendocrine cells. Except Paneth cells, newly formed epithelial cells move from the bottom of the crypt, along the villus-crypt axis to the tip of the villus, and are extruded after 4-5 days. During this process, the epithelial cells mature, but only when they reach the base of the villus, the full range of enzymes and other properties that are needed for their digestive and absorptive functions are acquired (Mowat & Agace, 2014).

Both villi and crypts play important roles in the digestion and absorption capacity of the small intestine. Notably, when the structure of the villus tip is impaired, or mature cells are lost during their migration, the reduced digestive surface area or increased presence of immature cells will lead to decrease of absorptive capacity.

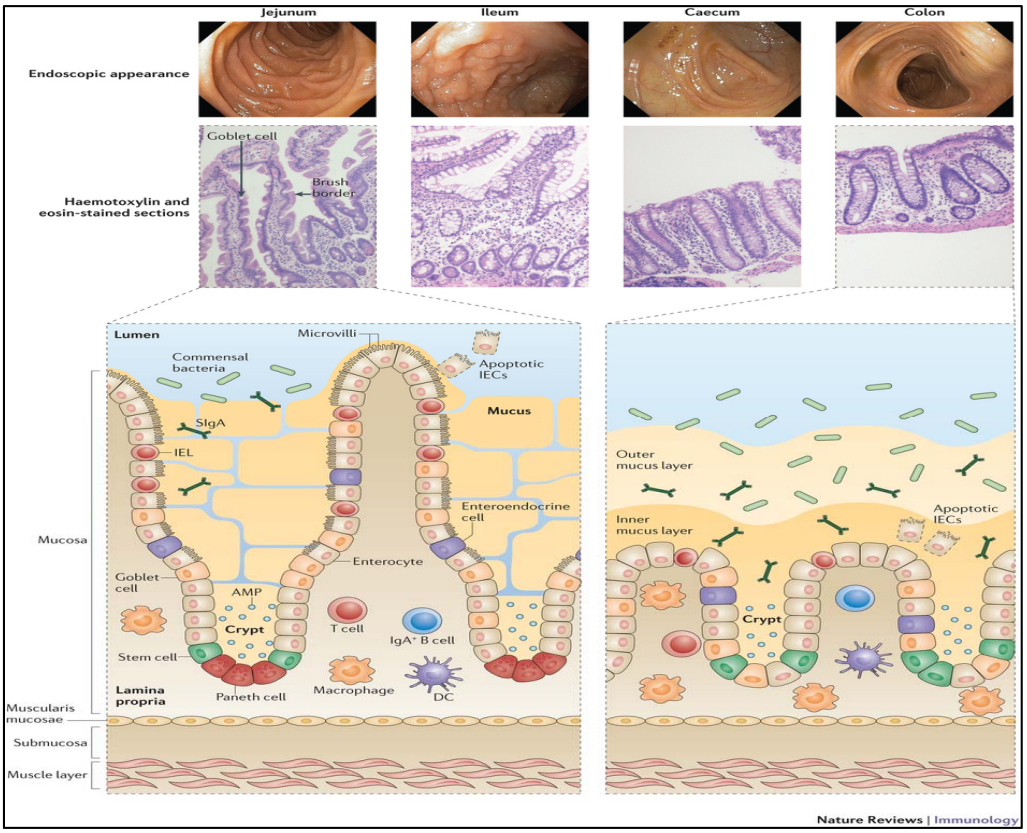

Figure I–2 *Anatomy of the intestinal mucosa (histological section and schematic) (Mowat & Agace, 2014)*

2.2 Small intestinal mucosal epithelial barrier function

The intestinal epithelium is a single-cell layer that constitutes the largest and most important barrier against the external environment (Groschwitz & Hogan, 2009). By forming complex protein-protein networks that mechanically link adjacent cells across the intercellular space, the intestinal epithelium maintains a barrier function which selectively permits the passage of nutrients, electrolytes, and water while performing defense against intraluminal toxins, antigens and enteric flora (Podolsky, 1999; Ferraris, 2001; Blikslager *et al.*, 2007; Broer, 2008; Groschwitz & Hogan, 2009). Also, this barrier function is highly regulated and dependent on the villus/crypt localization, and cell membrane specificity (apical, lateral, or basolateral) (Laukoetter *et al.*, 2006; Groschwitz & Hogan, 2009). Normally the intestinal epithelium mediates selective permeability through two major routes, i.e. the transepithelial (or transcellular) and paracellular routes (Tsukita *et al.*, 2001). The transcellular pathway is important for the intestinal endocytic uptake of luminal macromolecules. It includes simple passive diffusion, carrier mediated transportation (active transport and facilitated diffusion), and endocytosis. For the passive diffusion mechanism, the molecular must have the correct physical-chemical properties, such as size, charge, lipophilicity and hydrogen bonding properties to cross the lipophilic apical and basolateral membranes (Liu *et al.*, 2009). In the endocytosis pathway, macromolecules are mainly degraded by the enzymes in lysosomes or in the endocytic vesicles. Only a small amount of macromolecules can be released in intact form into interstitial space (Sanderson & Walker, 1993; Snoeck *et al.*, 2005). Generally, the transcellular permeability is associated with the action of specific transport channels for amino acids, electrolytes, short-chain fatty acids, and sugars (Ferraris & Diamond, 1997; Broer, 2008).

The paracellular permeability is associated with transport in the space between epithelial cells (Groschwitz & Hogan, 2009). In the presence of an intact epithelial cell layer, the paracellular pathway between cells is sealed, mediated by the apical junctional complex, which is composed of the tight

junctions (TJs) and subjacent adherent junctions (Turner, 2009). TJs are complex structures comprising a series of transmembrane proteins, cytoskeleton and plaque proteins (**Fig. I-3**). Pappenheimer and Reiss (1997) calculated that the effective pore size of intestinal epithelium is able to admit passage of solutes of 5 000 Da at the TJ . Thus, not only ions and water, but also small macromolecules could pass by the paracellular route (Atisook & Madara, 1991). Taken together, the regulation of intestinal barrier function is dependent, to a large extent, on the TJs permeability (Pacha, 2000; Turner, 2009). The transmembrane proteins containing occludin, claudins and junctional adhesion molecule (JAM) family protein, which seal the paracellular space between adjacent epithelial cells. The cytoskeletons are the intricate structures of protein filaments extending throughout the cytosol to maintain the structure of all eukaryotic cells. Plaque proteins, such as the zonula occluden (ZO) family are involved in the clustering and stabilization of the transmembrane proteins. Disruption in the composition of these proteins is linked to the loss of intestinal barrier integrity (Banan et al., 1999; Ulluwishewa et al., 2011). Additionally, the paracellular back flux of Na^+ could indirectly affect the transcellular pathway by influencing transcellular Na^+ -dependent absorption of nutrients (Madara, 1989).

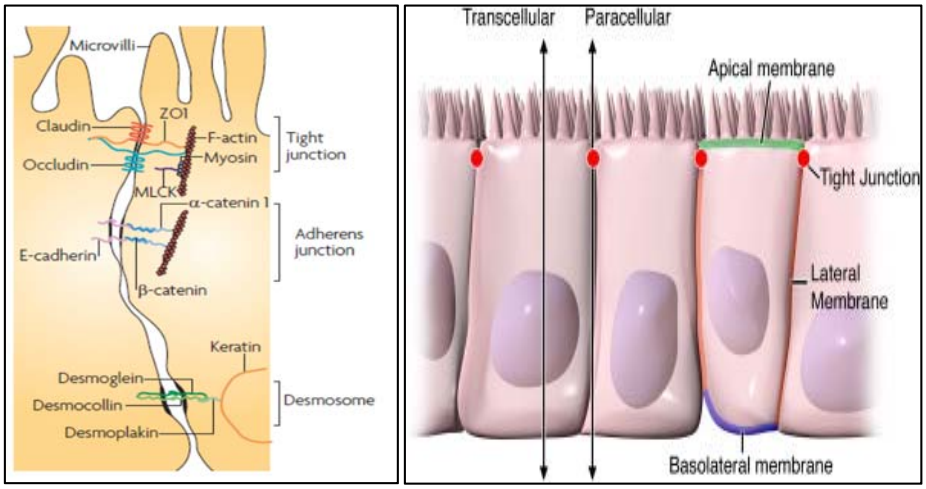

Figure I–3 Anatomy of the intestinal barrier and pathways of epithelial permeability (Groschwitz & Hogan, 2009; Turner, 2009)

2.3 Early development of the small intestine mucosa in pigs

In all parts of the intestine, the surface epithelium is continuously renewed by immature cells arising from crypts. Specifically, the development of the gastrointestinal tract (GIT) in the early stages of life is characterized by both remarkable morphological and functional changes (Zabielski *et al.*, 2008; Xu *et al.*, 2012). The birth and early neonatal period correspond to the change from parental to enteral nutrition. During the neonatal period, extensive changes occur in the gross architecture, ultrastructure, growth and differentiation of the intestine (Kelly & Coutts, 2000; Le Dividich & Seve, 2000; Trahair & Sangild, 2002). In piglets, increased intestinal mass (intestinal weight, length, diameter, mucosa weight villus height) (Xu *et al.*, 1992) and decreased rate of nutrient transport are seen in the first 24 h after birth (Puchal & Buddington, 1992; Zhang *et al.*, 1997). Also, rebuilding processes are noted in the epithelium during the neonatal period (Xu, 1996; Xu *et al.*, 2000; Zabielski *et al.*, 2008). By day 3 after birth, the density of villi decreases by 58% compared to the value at birth. At day 14, villi have irregular shape and the density is still significantly decreased (Skrzypek *et al.*, 2010). Only by day 21 after birth, the mucosa is covered by villi with a great diversity in shape, and the villus surface is covered by numerous cells including a large number of goblet cells (Skrzypek *et al.*, 2007a; Skrzypek *et al.*, 2010).

In addition to the progressive differentiation along the crypt-villus axis, the digestive and absorptive capacity of intestinal enterocyte also undergoes an age-related maturation process during suckling period (Fan, 2003). The activities of sucrase, maltase and maltase-glucoamylase are low in newborn piglets and increase to the 8th week postnatal. In contrast, to be able to digest the lactose in the milk, the lactase activity in the intestine of piglet at birth is higher than the level in fetal pig, and it reaches maximum during the first week postnatal (Aumaitre & Corring, 1978). Additionally, peptidase activities are generally high at birth and decrease during the suckling period (Zabielski *et al.*, 1999). As for lipase, it was stated that the pancreatic lipase activity to be low at birth, followed by a steady increase

in activity between day 7 and 14, and plateauing around day 48 postnatal (Dicklin *et al.*, 2006). Also, the fatty acid binding proteins (FABP) have been detected in the SI in newborn pigs, and the activity of FABP increases following the intake of colostrum (Reinhart *et al.*, 1992).

Notably, the transport of macromolecules is particularly important during early postnatal life since it facilitates the absorption of growth factors and immunoglobulin (Ig) G from maternal colostrum and milk (Pacha, 2000). This transport mechanism is crucial for pigs, as piglets are born nearly agammaglobulinemic, the transport of macromolecules make them capable of transferring intact Igs from ingested colostrum to the circulation during the early postnatal days (Westrom *et al.*, 1984). Except the specific receptor-mediated trancelluar to transport macromolecules, a unique feature of enterocytes in newborn mammals is the presence of an apical canalicular system, which can produce large vacuoles. These vacuolated equipped enterocytes play a key role in the nonspecific transcytosis to take up the colostrum macromolecules (immunoglobulins, hormones, growth factors, etc.). However, this kind of enterocytes are observed in the upper part of the villi only during the first 2-3 days of postnatal life in pigs (Skrzypek *et al.*, 2007b; Zabielski *et al.*, 2008). Therefore, the high permeability of the intestinal epithelium to macromolecules declines rapidly within the first 24-72 hours in piglets to avoid extra access of antigens(Guilloteau *et al.*, 2010). This process, which leads to decreased, even ceased uptake of macromolecules during early postnatal intestinal development is called gut closure (Walker, 1979; Westrom *et al.*, 1984; Pacha, 2000).

3 WEANING-INDUCED GASTROINTESTINAL DYSFUNCTION

Weaning under commercial condition is one of the most significant events in the pig's life because it involves complex psychological, social, environmental and dietary stresses (Lalles *et al.*, 2004). The weaning transition corresponds to a shift from maternal milk rich in protein, fat and special bioactive components like immunoglobulins, to a solid plant based diet. Therefore, the weaning process is accompanied by profound changes in the GIT of piglet as an adaptation to the diet. In commercial

farm conditions, weaning immediately leads to a dramatic reduction in feed intake, which in turn can affect gut structure and function and reduce overall growth rate (Hampson, 1986; Pluske *et al.*, 1997; McCracken *et al.*, 1999; Lalles *et al.*, 2004; Kim *et al.*, 2012). The most reported changes in mucosal architecture in weaning piglets are the reduction in villus height, concomitant with a hyperplasia in the crypt (Pluske *et al.*, 1997; McCracken *et al.*, 1999; Boudry *et al.*, 2002; Gu *et al.*, 2002). This effect on intestinal architecture is acute. It happens within 24 hours after weaning and is most pronounced by 3 to 5 days post weaning, particularly in the proximal SI (**Fig. I-4**) (Spreeuwenberg *et al.*, 2001; Pluske *et al.*, 2003).

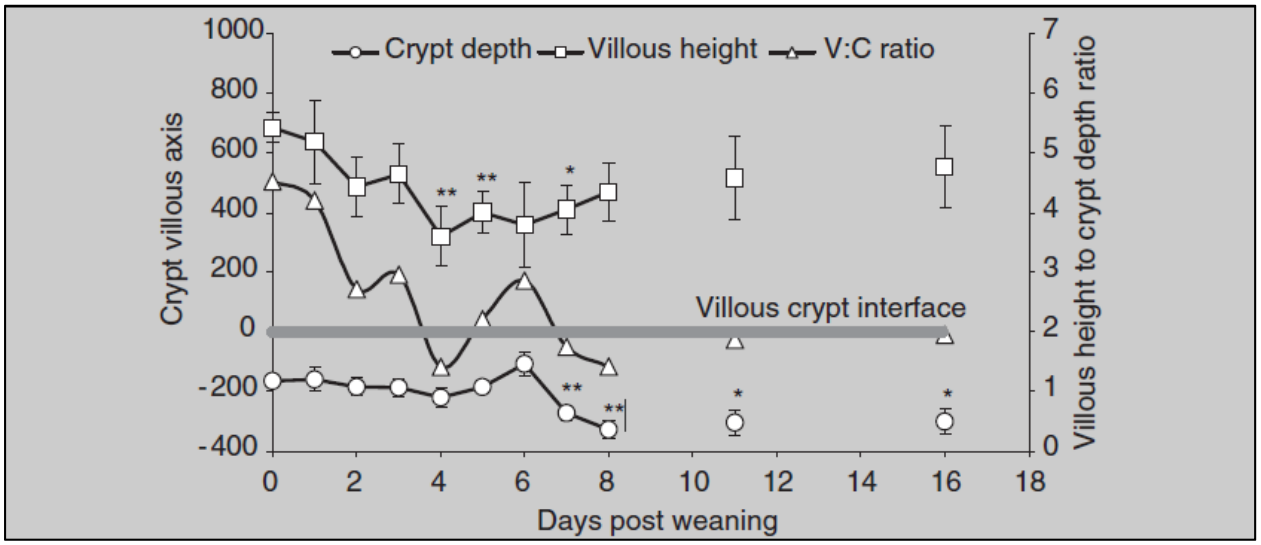

Figure I–4 *Changes in villus and crypt morphology following weaning (J.R. Pluske, 2003)*

Weaning also has significant effects on absorption and permeability properties in the small intestine (Lalles *et al.*, 2004) (**Fig. I-5**). The effect of weaning on intestinal permeability has been evaluated mostly *ex vivo* in Ussing chambers by measuring 1/ mucosal to serosal fluxes of small molecules (mannitol, fluorescein isothiocyanate dextrans), or tissue transepithelial electrical resistance (TEER) or the paracellular route and 2/ fluxes of macromolecules like horseradish peroxidase (HRP) or the transcellular route (Spreeuwenberg *et al.*, 2001; Boudry *et al.*, 2003; Blikslager *et al.*, 2007; Moeser *et*

al., 2007a; Moeser *et al.*, 2007b; Smith *et al.*, 2010). TEER is considered to vary in an opposite manner to the paracellular flux. Weaning age, weaning stress, feed intake and weaning diet composition are the four factors having a major effect on intestinal mucosal barrier permeability (Wijtten *et al.*, 2011). Notably, the paracellular pathway is consistently compromised after weaning, irrespective of the weaning age or diet composition, while the transcellular pathway for macromolecules through endocytosis decreases after weaning (Spreeuwenberg *et al.*, 2001; Wijtten *et al.*, 2011). This might be also an indication that the improvement of the transcellular pathway is a result of intestinal maturation, which could prevent the intestine from antigen overload.

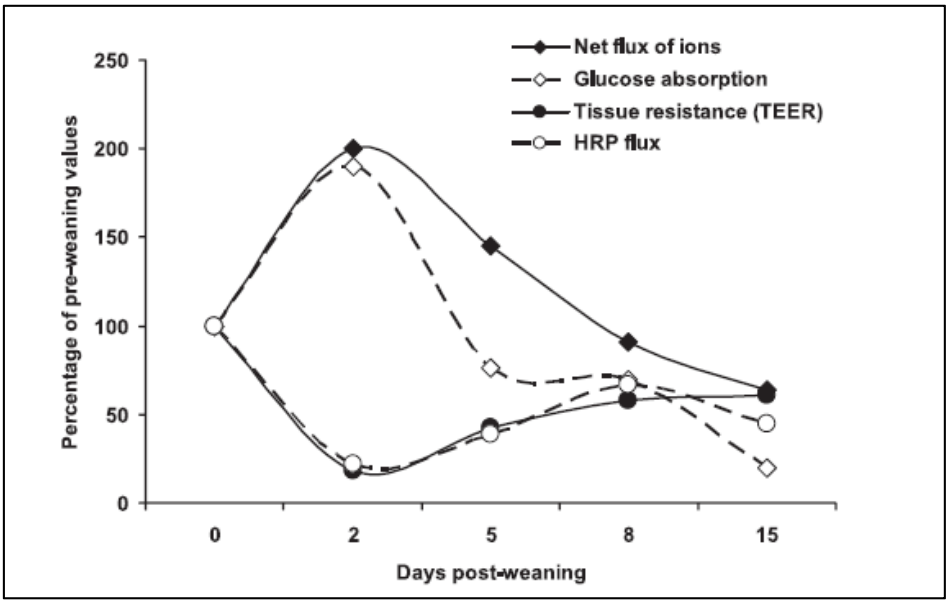

Figure I–5 *Influence of weaning on the net flux of ions, glucose absorption, tissue electrical resistance and horsradish peroxidase (HRP)flux in the proximal SI of piglets weaned at 21 days of age. The values are expressed as a percentage of preweaning values. (Lalles et al., 2004).*

4 INTRAUTERINE GROWTH RESTRICTION

4.1 Definition of intrauterine growth restriction

Intrauterine growth restriction (IUGR), or fetal growth restriction is defined as impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy. It is a noteworthy problem both in human neonatology and domestic animal production (Wu *et al.*, 2006). IUGR is

associated with high rates of perinatal morbidity and mortality (Rueda-Clausen *et al.*, 2011), a long-term stunting effect on postnatal growth and nutrient utilization (McMillen & Robinson, 2005; Wu *et al.*, 2006), and increased susceptibility to the development of various metabolic syndromes in adulthood (Ananth & Vintzileos, 2009).

4.2 Etiology of IUGR

Genetic, epigenetic, maternal, placental and environmental factors contribute to the occurrence of IUGR. As summarized by Bordsky and Christou (2004), the symmetric growth restriction is mainly caused by intrinsic factors like chromosomal abnormalities and congenital malformations, drugs, infection and early-onset severe preeclampsia. In contrast, the reason for asymmetric growth restriction is more extrinsic, including placental and maternal vascular factors, such as placental insufficiency . Next, maternal nutrition during pregnancy is an important determinant of subsequent fetal growth and development (Redmer *et al.*, 2004).

In domestic animals, IUGR is observed naturally in dams with multifetal pregnancies, due to the reduced placental mass per fetus and therefore relative placental insufficiency (Wootton *et al.*, 1983). Thus, uterine capacity affects the occurrence of IUGR (Dickinson *et al.*, 1962; Allen *et al.*, 2002). In pigs, the occurrence of IUGR originates mostly during late gestation, and the high rate of IUGR is likely a consequence of an imbalance between ovulation rate, and the uterine capacity due to ongoing selection for high litter size (Foxcroft et al., 2006). In addition, the differential growth of porcine fetuses is largely depending on the position in the uterus, next to uterine capacity (Perry & Rowell, 1969; Ashworth *et al.*, 2001). The pig has been suggested as a suitable model of IUGR (Bauer *et al.*, 1998b; Ashworth *et al.*, 2001).

4.3 Identification of IUGR

The easy assessment of fetal weight or birth weight relative to gestational age makes this a widely used criterion to detect IUGR (Wu *et al.*, 2006). In human clinics, the most common definition of IUGR is an estimated fetal weight less than the tenth percentile of the birth-weight-for-gestational-age reference curve according to ultrasound data (de Onis *et al.*, 1998). At term the cut off weight for IUGR infant is 2.5 kg (Cooper, 1975). However, it was noticed only a birth weight below 3rd percentile was stated a predictor of perinatal mortality (McIntire *et al.*, 1999). Therefore, another functional description, as a supplement to the definition of IUGR, is that a fetus who is unable to achieve its genetically determined potential size. This supplement allows to exclude fetuses which are small for gestational age but are not pathologically small.

In animal studies, IUGR is frequently identified as fetal or birth weight less than two standard deviations (SD) of the mean body weight of gestational age (Cooper, 1975; Wu *et al.*, 2008; D'Inca *et al.*, 2010a; D'Inca *et al.*, 2010b; D'Inca *et al.*, 2011). Of particular interest is that pigs exhibit the most severe, naturally occurring IUGR (Xu *et al.*, 1994; Wu *et al.*, 2006; Wang *et al.*, 2010). This high prevalence of IUGR in pigs poses an economic burden on piglet performance. Therefore, either from the perspective of using piglets as a model of IUGR for human infants (Cooper, 1975; Swanson & David, 2015), or from the perspective to improve the postnatal performance of pigs, piglets born with IUGR are worthy of attention.

Quiniou *et al.* (2002) investigated 12 041 piglets from 965 litters from 168 Large White × Landrace crossbred sows and reported that piglets can be classified as IUGR when the birth weight is between 0.6 and 1.0 kg, or smaller than the 11th percentile. However, Guilloteau *et al.* (2010) argued that the percentile classification could not be applied to a single litter, because birth weights do not follow a normal distribution. They suggested that classification of IUGR piglets must be based on the absolute value of birth weight for a certain breed, which is similar to the supplement of the functional

description of human IUGR definition. In a breeding study reported by Wu *et al.* (2010), birth weight less than 1.1 kg was used as criteria to identify IUGR piglets (Yorkshire × Landrace sows and Duroc × Hampshire sire), and these IUGR piglets represented 76% of preweaning deaths in that study. Most studies defined piglets born within 1 SD of the mean birth weight as normal birth weight piglets, whereas IUGR piglets are the ones having a birth weight up to 1 kg, or lower than 1.5 SD to 2 SD of the mean birth weight of the litter (Xu *et al.*, 1994; Wang *et al.*, 2008a; D'Inca *et al.*, 2010a; Wang *et al.*, 2010; D'Inca *et al.*, 2011; Wang *et al.*, 2014). It has been stated that litter size >15 have suffered from IUGR crowding, irrespective of the breed, because limited uterine capacity is unable to support a higher birth weight in these situations (Foxcroft, 2007). Bauer *et al.* (1998) also stated that animals with a body weight lower than the 10^{th} percentile were only found in litters with more than seven neonates. Thus, the litter size should be considered as well when identifying the IUGR piglets (Bauer *et al.*, 1998a).

4.4 Effect of IUGR on porcine gastrointestinal health

The GIT is of paramount importance in postnatal nutrient acquisition. In addition, the epithelial barrier is involved in the first steps of postnatal immune system maturation, protection against food antigens and invasion of environmental microorganisms (Xu *et al.*, 1994; D'Inca *et al.*, 2011). One feature of IUGR piglets is the impaired or retarded gastrointestinal development, which will further impose limitations on postnatal body growth and development of other organs (Xu *et al.*, 1994).

4.4.1 Effect of IUGR on porcine intestinal structure

Several studies have reported differences in intestinal architecture between IUGR and normal birth weight (NBW) newborns and during the neonatal period (**Table I-1**) (Xu *et al.*, 1994; Wang *et al.*, 2005; D'Inca *et al.*, 2010a; D'Inca *et al.*, 2010b; D'Inca *et al.*, 2011; He *et al.*, 2011; Wiyaporn *et al.*, 2013). The intestinal absorptive surface was smaller in IUGR during the first days of life in term piglets,

as indicated by reduced intestinal villus height and crypt depth. According to D'Inca *et al. (2010b),* this reduced surface of intestinal exchange probably results from a disturbed proliferation-apoptosis homeostasis . Additionally, this reduction of exchange surface might be crucial because of its important role in processing dietary molecules into available nutrients for the organism and in regulating the flux of antigenic material (D'Inca *et al.*, 2010b).

Table I–1 *Effect of IUGR on porcine intestinal structure*

Structure and physiological function		IUGR vs. Normal birth weight (NBW) piglets	References
At birth			
Length of small intestine relative to body weight Jejunal villus height, crypt depth Proximal ileal villus height, crypt depth Distal ileal crypt depth		↓	Xu *et al.,* 1994 Wang *et al.,* 2005 D' Inca *et al.,* 2010a,b& 2011
Neonatal period			
2-days age	Length of small intestine relative to body weight	↑	D'Inca *et al.,* 2010a, 2011
	Ileal villus area, villus height	↓	
5-days age	Length of small intestine relative to body weight	↑	
7-days age	Duodenal villus height/crypt depth ratio	↑	Wiyaporn *et al.,* 2013
10-days age	Proximal, middle and distal villus height, villus width, villus area and crypt depth	-	De Vos *et al.,* 2014 Huygelen *et al.,* 2015
21-days age	Jejunal villus height, villus height/crypt depth ratio	↓	Wang *et al.,* 2010 He et al., 2011
28-day age	Proximal, middle and distal villus height, villus width, villus area and crypt depth	-	De Vos *et al.,* 2014 Huygelen *et al.*, 2015

4.4.2 Effect of IUGR on porcine intestinal function

During the first days of neonatal period, the intestinal adaptation and bacterial colonization of IUGR piglets are altered, as well as an upregulation of barrier function interpreted as a protective response

against inflammation (D'Inca *et al.*, 2011). Proteome analysis of the jejunum of IUGR piglets showed that that the expression of key proteins involved in key biological processes, such as absorption, digestion and transport of nutrients; cell apoptosis; nutrient metabolism and cellular redox homeostasis and stress response is affected by IUGR during the first 21-days of life (Wang *et al.*, 2008a; D'Inca *et al.*, 2010b; Wang *et al.*, 2010). Moreover, He *et al.* (2011) reported that IUGR piglets have a distinctive metabolic status compared to NBW piglets on 21-day of age, whereby changes are related with lipogenesis, lipid oxidation, energy supply and utilization, amino acid and protein metabolism, and antioxidant ability. These results all suggest that during the neonatal period, IUGR is associated with both immediate and long-term altered intestinal adaptation in piglets.

5 OXIDATIVE STRESS & REDOX STATUS

5.1 Oxidative stress and redox status

For aerobic organisms oxygen is paradoxically both vital and inherently dangerous, since both the atomic and molecular form of oxygen may exist as free radical (Davies, 1995). During the reduction process of oxygen along the electron transport chain, oxygen-derived radicals, like superoxide anion, peroxide, hydroxyl radical, hydroxyl ion and reactive derivatives of oxygen (hydrogen peroxide, H_2O_2) are constantly generated, known as reactive oxygen species (ROS, **Fig. I-6**).

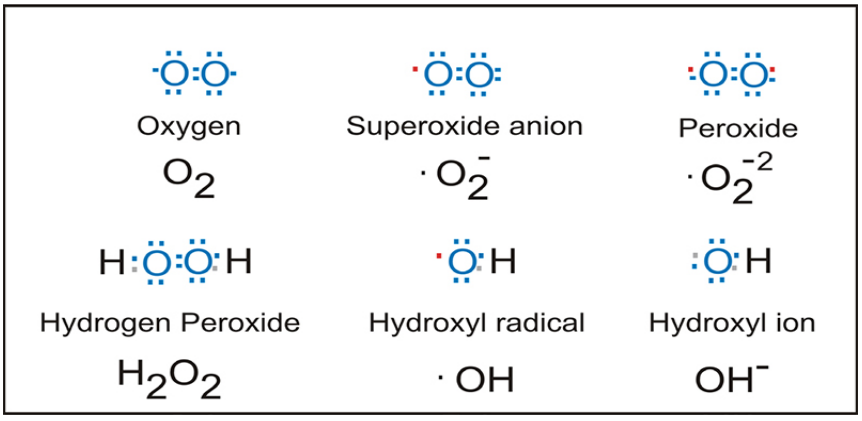

Figure I–6 *Electron structures of common reactive oxygen species. Each structure is provided with its name and chemical formula. The red • designates an unpaired electron. (Biotek.com)*

Regardless of how and where ROS are generated, a rise in intracellular oxidant levels may damage various cell components and trigger the activation of different signaling pathways (Finkel & Holbrook, 2000). In order to maintain proper levels of oxidant, a sophisticated enzymatic and non-enzymatic antioxidant defense system is present in cellular and extracellular compartments to counteract overall ROS levels (Finkel & Holbrook, 2000; Tauler Riera, 2012) (**Fig. I-7***).* The balance between oxidant/pro-oxidant production and antioxidants is defined as the redox status. Under healthy conditions, oxidants and antioxidants should be in equilibrium, i.e. redox homeostasis. When this homeostasis is disrupted, the equilibrium is tilted toward the oxidized state, which is referred to as oxidative stress (Davies, 1995; Finkel & Holbrook, 2000). Oxidative stress leads to damage to biological important molecules, such as proteins, lipids, carbohydrates and nucleic acids (DNA and RNA). Also, it has been implicated in a wide range of diseases, including cancer, heart attacks, chronic inflammatory diseases, and a wide variety of age-related disorders (Davies, 1995).

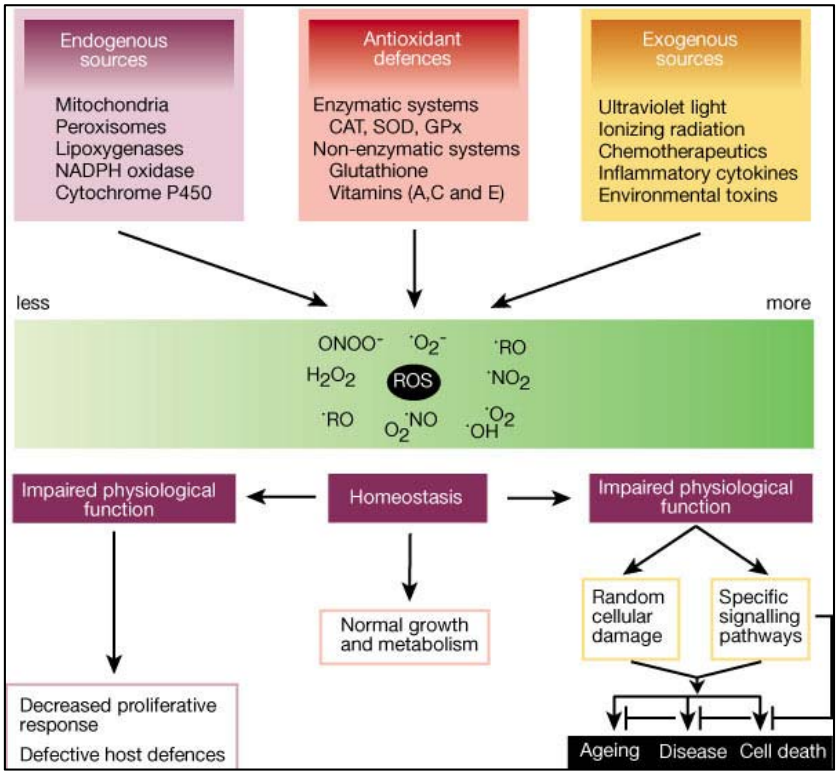

Figure I–7 *The sources and cellular response to reactive oxygen species (ROS) (Finkel & Holbrook, 2000). CAT, catalase; GPX, glutathione peroxides; SOD, superoxide dismutase.*

5.2 Intestinal antioxidant system

In addition to being exposed to luminal nutrients, the intestinal mucosal epithelium is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated reactive oxygen species that can contribute to oxidative stress (Ames, 1983; Aw, 1999). Oxidative stressis is considered an essential factor in the pathogenesis of gastrointestinal diseases, such as inflammatory bowel disease (IBD), enteric infection, colonic inflammation and cancer (Bhattacharyya *et al.*, 2014). To maintain integrity and proper physiological function, the intestinal mucosa is equipped with several antioxidant systems to defend against the harmful effects of ROS. The major enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione-disulfide reductase (GSR) and heme oxygenase (HO) (Thomson *et al.*, 1998; Bhattacharyya *et al.*, 2014). **Table I–2** summarizes the reaction these enzyme catalyzes with regarding to handle the ROS.

Beides these enzymatic antioxidants, the non-enzymatic antioxidant glutathione (GSH) is the most prevalent nonprotein cellular thiol present in cells. GSH acts as a reducing agent to detoxify various oxidants by acting as a coenzyme in GPX and glutathione S-transferase (GST) catalyzed reaction (Deneke & Fanburg, 1989; Wu *et al.*, 2004b; Iles & Liu, 2005). Notably, published results have shown that GSH is involved in intestinal mucosal peroxide transport, metabolism, cell turnover and oxidative susceptibility (Kowalski *et al.*, 1990; Aw *et al.*, 1992; Aw, 1994; Iwakiri *et al.*, 1995; Kanazawa & Ashida, 1998; Mohr *et al.*, 1999; Tsunada *et al.*, 2003; Aw, 2005). GSH concentration in cells is regulated at multiple aspects including de novo synthesis, redox cycling, cysteine availability, and consumption (**Fig. I-8**) (Iles & Liu, 2005). The synthesis of GSH is mainly controlled by glutamate cysteine ligase (GCL), previously known as γ-glutamylcysteine synthetase (γGCS), which catalyzes the first and rate-limiting step in the production of the cellular GSH. Dysregulation of GCL enzymatic

function and activity leads to decreased GSH biosynthesis, reduced cellular antioxidant capacity, and the induction of oxidative stress (Franklin *et al.*, 2009).

Table I–2 *Major intestinal enzymatic antioxidants*

Enzymes	Isoforms (location)	Reaction catalyzed and role in GIT disease
Superoxide dismutase (SOD)	- Cu-Zn-SOD (cytosol) Copper and zinc-containing enzyme - Mn-SOD (mitochondrial) Manganese-containing enzyme - EC-SOD Extracellular Cu-Zn containing enzyme	$Enz_{ox} + O_2^{\bullet-} \rightarrow Enz_{red}(H^+) + O_2$ $Enz_{red}(H^+) + O_2^{\bullet-} \rightarrow Enz_{ox} + H_2O_2$ Enhanced expression: prevent GIT mucosal injury, ulcer healing Reduced expression: cause gastric ulcer[1]
Glutathione peroxidase (GPX)	- GPX1 (ubiquitous) uniform crypt-to-villous distribution - GPX2 (GPX-GI, intestinal specific) crypt region predominating - GPX3 (extracellular) - GPX4	$2GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$ or $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$ GPX1 and GPX2 are major selenoproteins providing the first line of defense against ROS from inflammation[2] GPX1 and GPX2 double-knockout mice suffer from oxidative stress and inflammatory responses[3]
Glutathione-disulfide reductase (GSR)	Ubiquitously expressed	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ Protecting the cells by generating GSH[4]
Catalase (CAT)	Mainly found in peroxisomes	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ Not only detoxify H_2O_2, but also protect cells from apoptosis[5]
Heme oxygenase (HO)	- HO-1, the inducible isoform - HO-2, the constitutive isoform	$Heme + O_2 + NADPH + H^+ \rightarrow biliverdin + Fe^{2+} + CO + NADP^+ + 3H_2O$ HO-1 and its product CO have indirect cytoprotective response against oxidative stress, also is crucial in modulating cell apoptosis[6].

[1] Nenoi, et al. (2001), Kruidenier, et al. (2003).
[2] Chu, et al. (1997), Chow, et al. (1998).
[3] Esworthy, et al. (2001).
[4] Chang, et al. (1978).
[5] Nenoi, et al. (2001), Koutroubakis, et al. (2004).
[6] Vile, et al. (1994), Otterbein and Choi. (2000), Oates and West. (2006).

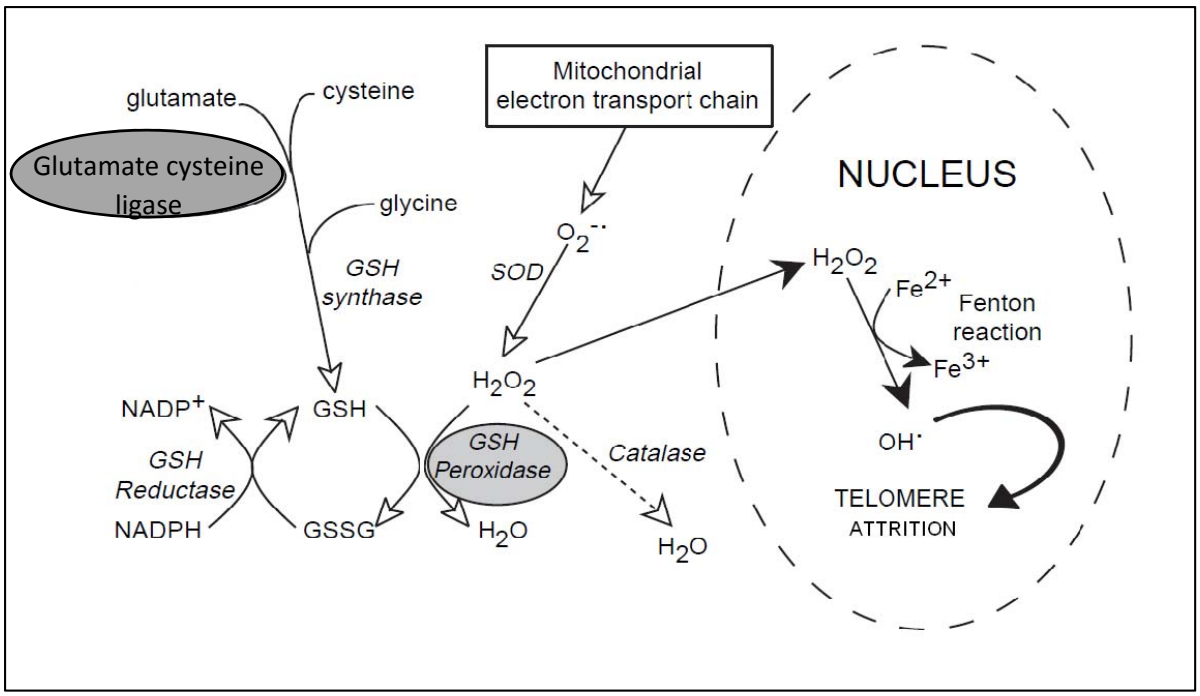

Figure I–8 *Glutathione redox cycle (Adapted from Kruz et al., 2004)*

5.3 Redox sensitive Signaling Pathways

ROS have been shown to be able to cause cellular damage, but they also function as signaling molecules that are necessary to activate a number of cellular pathways involved in cell proliferation, survival and inflammation (Hancock *et al.*, 2001; Fuh *et al.*, 2002; Schieber & Chandel, 2014). In this process, transcriptional factors like mitogen-activated protein kinases (MAPK), nuclear transcription factor kappa-B (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf2) or activator protein-1 (AP-1) are activated, subsequently regulating downstream gene expression (Sporn & Liby, 2012). On the other hand, the activation of NF-κB or Nrf2 can increase the transcription of antioxidant scavengers, such as *SOD*s, thioredoxin 1&2, glutathione *S*-transferase (*GSTs*), heme oxygenase-1 (*HMOX1*) and *GPX1* to keep a proper ROS level (Meyer *et al.*, 1993; Lavrovsky *et al.*, 1994; Xia *et al.*, 1996; Huang *et al.*, 2000; Djavaheri-Mergny *et al.*, 2004; Rojo *et al.*, 2004; Schreiber *et al.*, 2006; Kairisalo *et al.*, 2007; Morgan & Liu, 2011).

To note, substantial evidence has revealed that certain amounts of ROS are required for the release of pro-inflammatory cytokines of interleukin 1β(IL-1β), tumor necrosis factor-α (TNF-α) and Interferon-β (IFN-β), thus promote proper immune response (Kong *et al.*, 2011; West *et al.*, 2011). Slight elevation of ROS levels even could enhance immune system function, but excessive production of ROS can lead to pathological responses (Schieber & Chandel, 2014). Importantly, the intestine disposes of injured or spent enterocytes by inducing cell death to maintain the mucosal integrity (Aw, 1999). Importantly, studies with tumor necrosis factor (TNF)-mediated inflammation have linked TNF-induced intestinal barrier dysfunction, apoptosis, and oxidant production with the signal transduction of TNF-induced NF-κB activation (Chandel *et al.*, 2000; Ma *et al.*, 2004; Han *et al.*, 2006; Overman *et al.*, 2012).

5.4 Oxidative stress and IUGR

Preeclampsia in pregnant woman has been associated with maternal oxidative stress (Davidge *et al.*, 1992; Mutlu-Turkoglu *et al.*, 1998). Since preeclampsia with placental insufficiency and ischemia, is one of the etiological factors of IUGR, oxidative stress is thought playing an important role in IUGR. Biri *et al.* (2007) reported that oxidative stress biomarkers like malondialdehyde (MDA) were higher, and antioxidant potential represented by SOD and GPX were lower in maternal plasma, cord plasma and placenta tissue of IUGR newborns (Biri *et al.*, 2007).

In pigs, recent proteome and metabolomics analyses revealed that the IUGR continuously affects the redox homeostasis in porcine fetuses, newborns and during the first three weeks of life (Wang *et al.*, 2010; He *et al.*, 2011). In addition, in case of adequate nutrient intake during the neonatal period, increased serum MDA level and lower concentration of α-tocopherol, as well as lower hepatic GPX activity and mRNA level of *SOD2* were found in 21-d old IUGR piglets, compared to the age-matched NBW ones (Che *et al.*, 2015), indicating a deteriorated antioxidant system in IUGR piglets. In weaned

piglets, it was shown that IUGR piglets had lower antioxidant capacity in plasma (Michiels *et al.*, 2013). Also, weaned IUGR piglets exhibited increased ROS level and lower efficiency of GSH in hepatocytes, compared to the age-matched NBW piglets, indicating the susceptibility of increased oxidative stress in IUGR offspring (Zhang *et al.*, 2014).

Table I–3 *Effect of IUGR on redox-sensitive protein/peptide expression*

Stage	Tissue	Redox-sensitive protein/peptide (IUGR *vs.* Age-matched NBW ones)	IUGR vs. NBW	Reference
Fetus in mid gestation (D60)	Jejunal mucosa	- Peroxiredoxin-1&2	↑	Wang *et al.*, 2014
Fetus in late gestation (D90)		- Peroxiredoxin-1&2, - Glutathione *S*-transferase	↑	
Fetus in late gestation (D110)		- Peroxiredoxin-1&2, - Glutathione *S*-transferase	↑	
Fetus in late gestation (D110)	Liver	- Alpha-1 acid glycoprotein - Liver fatty acid binding protein	↑	Liu *et al., 2013*
		- Glutathione *S*-transferase alpha M14 - Dimeric dihydrodiol dehydrogenase - Alternative pig liver esterase	↓	
Neonate (1-d old)	Liver	- Peroxiredoxin-1 - Transferrin - ζ-crystallin	↓	Wang *et al.,* 2008
	Skeletal muscle	- Peroxiredoxin-1	↓	
	Jejunal mucosa	- Beta-globin	↓	
		- Glutathione*S*-transferase omega	↑	Wang *et al.*,2010
7d-old	Jejunal mucosa	- Glutathione *S*-transferase omega - Peroxiredoxin-5 - Heat shock 70 kDa protein 8	↑	
21d-old	Jejunal mucosa	- Glutathione *S*-transferase omega - Peroxiredoxin-5 - Heat shock 70 kDa protein 8	↑	
		- Protein disulfide isomerase associated 3	↓	
	Jejunum	Glutathione[1]	↑	He *et al.*, 2011

[1] Detected by metabolomics analysis.

6 CONCLUSION

Dramatic shifts of intestinal epithelial function occur in the early life of animals, and weaning induces additional both transient and long-term changes in intestinal physiology. However, little is known about the effect of weaning in IUGR on structural and functional parameters of the GIT. Nevertheless, this point appears to be crucial because the GIT is involved in the first steps of immune system maturation, in body protection against food antigens, environmental microorganisms and in nutrient assimilation. To our knowledge, most studies of IUGR piglets focused on investigating the effect of IUGR at birth or during the early postnatal period, but whether IUGR will have a long-term effect on intestinal heath, and whether weaning will exert extra stress in IUGR piglets is poorly explored. In addition, emerging evidence reveals that there is a significant difference in the GIT development between normal and IUGR piglets at birth, represented by retarded growth, impaired barrier function, immune response or energy metabolism. Although the observations are of considerable importance for the link between intestinal physiology and IUGR offspring, this has not received sufficient attention. Whether the significant shift during early postnatal phase will make a change to the oxidative and redox status of the intestine, and whether IUGR piglets will exhibit a disordered intestinal development and function in this respect is still incompletely understood.

Chapter II

Effect of intrauterine growth restriction on the intestinal barrier function, redox status and redox-sensitive genes expression during the neonatal period of piglets

Adapted from: Wang, W., Degroote, J., Van Ginneken, C., Van Poucke, M., Vergauwen, H., Dam, T. M., Vanrompay, D., Peelman, L. J., De Smet, S., & Michiels, J. (2016). Intrauterine growth restriction in neonatal piglets affects small intestinal mucosal permeability and mRNA expression of redox-sensitive genes. FASEB J, 30(2), 863-873.

ABSTRACT

Neonates with intrauterine growth restriction (IUGR) show lower efficiency of nutrient utilization compared to normal birth weight (NBW) newborns. This study was conducted using neonatal piglets as model to test the hypothesis that IUGR affects the intestinal barrier function, intestinal structure and antioxidant system development during the neonatal period. The small intestinal mucosae were obtained from IUGR and NBW littermates in the neonatal period (day 0, 3, 8 and 19 postnatal, DPN0, 3, 8, 19). The epithelial barrier function was assessed by fluorescein isothiocyanate-dextran (FD4) and horseradish peroxidase (HRP) fluxes across the epithelium, histo-morphological measurements, and expression of tight junction proteins. Redox status represented by the glutathione disulfide/glutathione ratio and malondialdehyde concentrations were determined, while mRNA levels of some redox-sensitive proteins were quantified. Results showed that IUGR piglets exhibited a two-fold higher intestinal permeability in the proximal SI on DPN0 ($P < 0.05$), and this difference between IUGR and NBW piglets was widened to 3 and 4 times for FD4 and HRP, respectively ($P < 0.05$) on DPN3. In accordance, expression of occludin was down regulated at transcriptional level in IUGR piglets at DPN0 and DPN19 ($P < 0.01$). Furthermore, the transcription of *heme oxgenase 1*, *catalase* and *thioredoxin reductase* genes were down regulated in IUGR piglets, mainly on DPN0 and DPN19 postnatal ($P < 0.01$). It appears that IUGR subjects have lower capacity to mount an antioxidant response in the early postnatal period. Collectively, these results add to our understanding of the mechanisms responsible for intestinal dysfunction in IUGR neonates.

1 INTRODUCTION

Intrauterine growth restriction (IUGR) is defined as impaired growth and development of the mammalian embryo/fetus or organs during pregnancy, which results in a fetal or birth weight less than two standard deviations of the mean body weight at the corresponding gestational age (Wu *et al.*, 2006; D'Inca *et al.*, 2010a). Neonates with IUGR show substantially higher rates of perinatal mortality and morbidity, partly due to impairment of cell proliferation, and nutrient digestion, absorption and metabolism in the small intestine (SI) (D'Inca *et al.*, 2011; Mickiewicz *et al.*, 2012; Ferenc *et al.*, 2014).

Pigs exhibit the most severe naturally occurring IUGR among mammalian species (Wu *et al.*, 2006). Due to the structural, developmental and dietary similarities, the pig model has been chosen as the most optimal one for studying gastrointestinal physiology and function in human IUGR syndrome (Wang *et al.*, 2008a; Ferenc *et al.*, 2014). In a recent study using proteomics, IUGR neonatal piglets exhibited altered expression of a number of proteins involved in the cell redox homeostasis in the SI (Wang *et al.*, 2010). The expression of beta-globin was reduced in intestine from IUGR as compared to normal birth weight (NBW) piglets, which may contribute to a defect in oxygen transport and, therefore, intestinal ischemia which could affect the cell redox homeostasis (Wang *et al.*, 2010). Further, peroxiredoxin-1 and -5, important antioxidant enzymes, together with glutathione S-transferase, were also higher expressed confirming a disturbed redox status. Finally, these researchers found a reduction of protein disulfide isomerase-associated 3, indicative for attenuation of redox-sensitive signaling pathways. However, the results were inconsistent across time points postnatal (DPN1, DPN7 and DPN21). Additional evidence was provided by the same group in an earlier paper, where they demonstrated upregulation of scavenger-receptor protein in newborn IUGR piglets (Wang *et al.*, 2008a). On transcriptional level, oxidative stress has been shown to modulate redox-sensitive transcription factor NF-E2 related factor-2 (Nrf2), nuclear factor-kappa B (NF-κB) or activator protein-1 (AP-1), thereby fortifying cellular antioxidant capacity or suppressing inflammation (Surh *et*

al., 2005). Taken together, compromised redox homeostasis and redox-sensitive signaling in the SI may happen in IUGR subjects after birth.

The newborn is particularly susceptible to O_2-derived free radicals, since at birth a neonate abruptly enters a hyperoxic extrauterine environment, with being exposed to an O_2 tension approximately five times greater than that during intrauterine development (Robles *et al.*, 2001; Friel *et al.*, 2004; Yin *et al.*, 2013). The antioxidants transferred to the neonate during late gestation may not be sufficient to clear the excessive generation of free radicals, leading to oxidative stress (Robles *et al.*, 2001; Friel *et al.*, 2004). To preserve cellular integrity and tissue redox homeostasis, the intestine possesses several antioxidant defense systems such as maintaining high antioxidant concentrations (e.g. glutathione, GSH) and antioxidant enzymes systems (e.g. glutathione peroxidase, GPX; catalase, CAT and superoxide dismutase, SOD) (Aw, 2005). Oxidative stress at birth has been shown to affect the redox status and antioxidant system of the SI of piglets during neonatal period (Yin *et al.*, 2013). In addition, several studies with rodents showed that intestinal oxidative stress leads to an increase in intestinal paracellular permeability (Darmon *et al.*, 1993; Maeda *et al.*, 2010; Dixit *et al.*, 2012), by rupture of the intestinal barrier and/or by reduction of the expression of the redox-sensitive membrane-cytoskeleton protein occludin (Rao *et al.*, 2002; Sheth *et al.*, 2003).

After birth, intensive development of the SI is induced by the shift from parenteral to enteral nutrition. These adaptations take place continuously during the whole neonatal period (Zabielski *et al.*, 2008). Differences in gastro-intestinal development between IUGR and normal birth weight (NBW) neonates have been extensively documented. However, it is still unclear whether the intestinal dysfunction lasts during the early life of IUGR subjects, and the effect of IUGR on the redox homeostasis of SI is unknown. In the present study, it was hypothesized that IUGR has an effect on the intestinal barrier function, and is accompanied by disturbed responses to birth oxidative stress during neonatal period. To test this hypothesis, the intestinal barrier function was assessed *ex vivo* by measuring the

permeability of markers with different molecular weights and the expression of TJs proteins, and their association with inflammatory responses. Also, markers of oxidative stress, components of the GSH redox cycle, the antioxidant system, both on transcriptional and translational level were analyzed during the 3-week nursing period of IUGR and NBW littermates.

2 MATERIALS & METHODS

2.1 Pig model and tissue collection

The experiment was carried out according to the guidelines of the Ethical Committee of Ghent University (Belgium) for the humane care and use of animals in research (Nr. EC2011/195). Twenty-four pairs of piglets (Topigs hybrid x Piétrain) were selected from twenty-three sows in one farm with a four-week farrowing system, during two consecutive farrowing rounds. No cross-fostered piglets were included in the experiment. Full term newborns were weighed within 12 hours after parturition to determine birth weight and gender. Piglets were individually tagged with a unique ear tag and birth weight was used as criterion to identify IUGR and NBW littermates. An IUGR piglet was defined as piglet having a birth weight between 0.75 and 0.90 kg in a litter with 14 or more live-born piglets, and belonging to the lower quartile of the litter birth weights, while a NBW littermate had a birth weight within ± 1 standard deviation of the mean birth weight of the whole litter. In the current study, average birth weight for all IUGR and NBW piglets in the study was 0.81 ± 0.02 and 1.30 ± 0.03 kg, respectively. All piglets were able to suckle the sow until sampling. Six couples of IUGR and NBW gender-matched littermates were sampled on days 0, 3, 8 and 19 postnatal (DPN0, 3, 8, 19). Piglets sampled on DPN0 were removed from the sow between 12 and 24 h after parturition. Colostrum intake was verified by visual check and palpation, especially for the IUGR ones. All the selected animals did not receive any antibiotic treatment prior or during the experiment.

Prior to removing the gastro-intestinal tract (GIT), piglets were killed by exsanguination following induction of terminal anesthesia by intra-peritoneal sodium pentobarbital at a dosage of 90 mg/kg

body weight (Dolethal®, Vétoquinol S.A., Aartselaar, Belgium). The SI, defined as the part of the GIT between the pylorus and the ileo-cecal valve, was obtained and its length was measured. A 10 cm segment of proximal and distal SI (5% and 75% of total SI length) was taken for Ussing chamber measurements. In addition, 20 cm segments at 5% or 75% of the total SI length were emptied and carefully flushed with saline. The tissue of these 20 cm segments was placed on an ice-cold surface and the mucosa was retrieved by gently scraping the mucosal surface with a glass slide. Then, the mucosa scraping was transferred to a glass beaker and stirred with a spoon to obtain a homogeneous representative sample. Aliquots of the mucosa were either used instantaneously for acid and phosphate buffered aqueous extracts or transferred to plastic 2 mL screw-capped tubes, snap-frozen in liquid nitrogen and stored at -80°C pending gene and protein expression analysis. Finally, 10 cm segments at 5% or 75% of the total SI length were taken, flushed with saline, fixated in 10% formaldehyde buffer and paraffin-embedded for histo-morphological and cell density measurements.

2.2 *Ex vivo* measurement of intestinal permeability

Intestinal mucosal permeability was assessed ex vivo by measuring the translocation of macromolecular markers using the Ussing chamber technique. Fresh segments of proximal or distal SI were first rinsed with saline, then collected in duplicate, stripped from the seromuscular layer, pinned onto 1.07 cm^2 sliders and mounted into modified Ussing chambers (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany). All tissues were mounted within 15 minutes following euthanasia. Tissues were surrounded by 6.5 mL Ringer solution (115 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L $NaHCO_3$, 2.4 mmol/L Na_2HPO_4, 0.4 mmol/L NaH_2PO_4, 1.25 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$) with 6 mmol/L of mannitol or glucose in the luminal and serosal side, respectively. The system was water-jacketed to 37 °C and oxygenated with 95% O_2 and 5% CO_2 gas flow. After an equilibration period of 20 min, 4 kDa fluorescein isothiocyanate-dextran (FD4, Sigma-Aldrich, Bornem, Belgium) and 40 kDa horseradish peroxidase (HRP, type IV, Sigma-Aldrich, Bornem, Belgium) were added to the

mucosal side to a final concentration of 0.8 mg/mL of FD4 and 0.4 mg/mL of HRP. Samples from serosal side were taken at 20, 40, 60 and 100 min after adding markers. Meanwhile, the same volume of Ringer solution was also taken from the mucosal side to keep the volume balance across sides. Fluorescence intensity of FD4 in the medium was measured at excitation wavelength of 485 nm and emission wavelength of 538 nm using a fluorescence plate reader (Thermo Scientific, Marietta, OH, USA). Enzymatic activity of HRP was measured according to the method described previously (McKie *et al.*, 1999).

The apparent permeation coefficient (Papp) was calculated as: Papp (cm/s): (dc/dt) × V/c0/A, whereby dc/dt is the change of serosal concentration in the 20- to 100-min period; V is the volume of the chamber, c0 is the initial marker concentration in the mucosal reservoirs and A is the area of the exposed intestine in the chambers (cm^2).

2.3 Tissue homogenate extracts and biochemical assays

First, total protein content was determined with the biuret method. Mucosal GSH and GSSG were measured using a modified high performance liquid chromatography (HPLC) method (Reed *et al.*, 1980; Degroote *et al.*, 2012; Vergauwen *et al.*, 2015). Briefly, an acid extract was prepared by approximately 1 g of homogenized mucosa in 10 mL ice-cold perchloric acid (PCA), 10 % solution with a Braun homogenizer at 900 rpm. This homogenate was then centrifuged at 15,000 G for 15 min at 4 °C. After that, 0.5 mL of the resulting acid extract was transferred to tubes containing 50 µL γ-glu-glu internal standard solution. Samples were then snap frozen in liquid nitrogen and stored at -80 °C until analysis of GSH and glutathione disulfide (GSSG).

The derivation procedure includes the reaction of 100 mmol/L iodoacetic acid solution with thiols to form S-carboxymethyl derivatives followed by chromophore derivation of primary amines with dinitrofluorobenzene (DNFB, 1% (v/v) in ethanol). Then, GSH and GSSG were separated through

HPLC (Agilent 1200 series system, equipped with degasser, auto sampler, EC250/4.6 Nucleosil 120-7 NH_2 aminopropyl column (Machery-Nagel, Düren, Germany) protected by the same NH_2 guard column (CC8/4), quaternary pump, column oven and UV detector). Chromatographic runs were performed at a flow-rate of 1.5 mL/min, starting at 80% solvent A / 20% solvent B for 5 min followed by a 10 min linear gradient to 1% solvent A / 99% solvent B and a 10 min isocratic period at 1% solvent A / 99% solvent B (solvent A: water-methanol solution (1:4, v/v), solvent B: 0.5 mol/L sodium acetate–64% methanol). The column was then re-equilibrated to the initial conditions for 15 min. The column temperature was maintained at 40°C. The UV detector was set at 365 nm for absorption measurements. GSH and GSSG were identified by retention times of authentic standards. Concentrations were determined by using the internal and external standards and expressed as µmol/g protein.

In addition, a phosphate buffered aqueous extract was made by mixing approximately 1 g of homogenized mucosa in 10 mL ice cold 1% Triton-X-100 phosphate buffer solution (pH = 7.0), by using an Ultra-Turrax dispensing machine (IKA-Werke GmbH & Co. KG, Staufen, Germany). The supernatant was then transferred to 2mL tubes, snap frozen and stored at -80 °C until analysis. The thiobarbituric acid reactive substances (TBARS) method was employed and expressed as malondialdehyde (MDA) concentrations to assess lipid peroxidation (Grotto *et al.*, 2007).

2.4 Histo-morphological and cell density measurements

To further substantiate the differences found in functional permeability at 5% of SI length at DPN0 and DPN3, histo-morphological and cell density measurements were performed. Histo-morphological variables were determined according to De Vos et al. (De Vos *et al.*, 2014). In brief, of paraffin-embedded samples 4 µm sections were mounted on slides and stained with hematoxylin-eosin. Villus height, mid-villus width, and crypt depth were measured at 10× magnification using an Olympus

BX61 microscope and analySIS Pro (Olympus Belgium, Aartselar, Belgium) in 30 well-oriented villi and associated crypts. Villus surface area was calculated by the following equation:

$$\text{Villus surface area} = 2\pi \times (\text{villus width}/2) \times \text{villus height}$$

The volume density (Vv) of the cell nuclei in the lamina propria mucosae was estimated using the CastGrid image analysis software (Olympus Belgium, Aartselaar, Belgium) in 4 µm paraffin sections that were stained with haematoxylin-eosin. A point grid at magnification 200x. The following equation was used to calculate the Vv (cell nuclei, lamina propriae mucosae):

$$Vv = \sum P(\text{cell nuclei}) / \sum P(\text{lamina propriae mucosae})$$

Where ∑P (cell nuclei) is the number of points of the counting grid that hit cell nuclei and ∑P(lamina propria mucosae) is the number of points hitting the lamina propria mucosae. The optimal density of the stereological grid (number of points), the number of sections and the number of fields were estimated as described previously (Gundersen & Jensen, 1987) and resulted in analysing approximately 30 fields of vision in at least 15 systematic random sections of each tissue block.

2.5 RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Because major differences in permeability were found at the proximal SI, gene expression was performed on proximal intestinal mucosa samples. Mucosal total RNA was isolated from snap-frozen intestinal mucosa at 5% of SI length, using Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, CA, USA), including an on-column DNase treatment to remove genomic DNA (gDNA). The concentration (ranging between 250-1200 ng/µL) and purity (OD 260/280 ranging between 1.9-2.2) of RNA was measured with NanoDrop ND-1000 (Nanodrop Technologies, Thermo Scientific, Wilmington, DE, USA). Integrity of RNA was verified by loading RNA onto a 0.8% agarose gel and evaluating the 28S and 18S ribosomal RNA bands, while verification of the absence of any gDNA contamination by means of a minus reverse transcription control PCR

using YWHAZ primers (**Table II–1**). Then 1 µg of high-quality gDNA-free RNA from each sample was converted to cDNA in the subsequent 20 µL RT reaction with ImProm-II cDNA synthesis kit (Promega, Madison, USA), containing both oligo dT and random primers. The cDNA was diluted 10 times with molecular grade water before verification of the reverse transcription reaction through a control PCR using 2 µL cDNA and the same YWHAZ primers as mentioned above.

Primers used for the target genes in the present study were designed with PRIMER3PLUS (Untergasser *et al.*, 2007), based on the certain exon-exon boundaries of published pig gene sequences, to amplify the specific isoforms of the genes listed in **Table II-1**. The mutations and the secondary structure in target sequence was checked with RepeatMarker and mFold (Zuker, 2003), respectively. The RT-qPCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 2 µL of cDNA template was added to a total volume of 10 µL containing 2× KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosystems, Inc, Wilmington, MA, USA) and 0.5 µmol/L each of forward and reverse primers. The following protocol was used for RT-qPCR, (i) enzyme activation and initial denaturation (95°C for 3 min); (ii) denaturation/annealing/extension and data acquisition, repeated 40 cycles (95°C for 20 s, 40 s at annealing temperature depending on primer), (iii) melt curve analysis from 70°C to 90 °C with 0.5°C increment every 5 seconds. A five-fold dilution series (5 points) of cDNA was included in each run to determine PCR efficiency by constructing a relative standard curve. In this study, PCR efficiencies were consistently between 95%-108% and were used to convert the Cq-values into raw data. The relative expression was expressed as a ratio of the target gene to the stable expressed reference gene, then the highest expressed samples were used as calibrator for the normalization of raw data.

Table II–1 *Primer sequences used for RT-qPCR* [*]

Gene Symbol	Accession NO.	Nucleotide sequence of primers (5'-3')	Product length (bp)
ACTB	XM_003124280.3	F: TCTGGCACCACACCTTCT R: TGATCTGGGTCATCTTCTCAC	114
HMBS	DQ178125	F: CTGTTTACCAAGGAGCTGGAAC R: TGAAGCCAGGAGGAAGCA	100
HPRT1	DQ178126	F: CCGAGGATTTGGAAAAGGT R:CTATTTCTGTTCAGTGCTTTGATGT	181
TBP	DQ178129	F: GATGGACGTTCGGTTTAGG R: AGCAGCACAGTACGAGCAA	124
TOP2B	NM_001258386.1	F: ATGCAACCAACACATCCTATC R: GCATTATTAGCGTGCTGTCTT	137
YWHAZ	DQ845179	F: AACTGGATGATGCTAATGATGCT R: TGGAAAAACTCCGTATCTGTCTC	178
RPL4	XM_005659862	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122
PPIA	NM_214353	F: CTGAAGCATACGGGTCCTGG R: TGCCCTCTTTCACTTTGCCA	139
OCLN	NM_001163647.2	F: CATGGCTGCCTTCTGCTTCATTGC R: ACCATCACACCCAGGATAGCACTCA	129
HP (zonulin)	NM_214000.2	F: TGATTTTCCATCCTGACAACTCCAC R: CCCACAAGCCCCACATTCAC	130
SOD1	NM_001190422	F: GGTCCTCACTTCAATCCTGAATCC R: CACACCATCTTTGCCAGCAGT	102
GPX1	NM_214201.1	F: TGCTCATTGAGAACGTAGCGT R: CAGGATCTCCCCATTCTTGGC	161
CAT	NM_214301.2	F: TGGACACAGGCACATGAACGGATAT R:CTTGCTGCATCTTCAACGGAAAGGT	132
PPARG	NM_214379.1	F: TCCAGCATTTCCACTCCACAC R: GGGACACAGGCTCCACTTTG	127
VNN1	NM_214133.1	F: TGAAGCCACCGTCTGGACCTGTCT R:CCTTGTGCCACGAGGTCTGCTGAAG	100
TXNRD1	NM_214154.3	F: GTGCTGAGGAGCTTCCCGAGATGT R:TCCAGGACCATGACCCGCTTGTTAA	118
HMOX1	NM_001004027.1	F: CGCTCCCGAATGAACACTCT R: GCGAGGGTCTCTGGTCCTTA	148
TNF-α	NM_214022.1	F: CATGATCCGAGACGTGGAGC R: AACCTCGAAGTGCAGTAGGC	151

[*] F, forward primer; R, reverse primer; ACTB, β-actin; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; TBP, TATA box binding protein; TOP2B, topoisomerase (DNA) II beta; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta; RPL4, ribosomal protein L4; PPIA, peptidylprolyl isomerase A; OCLN, occludin; HP, haptoglobin (alias:zonulin); SOD1, Copper/zinc superoxide dismutase; GPX1, glutathione peroxidase 1; CAT, catalase; PPARG, peroxisome proliferator-activated receptor gamma; VNN1, Vanin-1; TXNRD1, thioredoxin reductase; HMOX1, heme oxygenase 1; TNF-α, tumor necrosis factor alpha. All these primer sequences were designed based on the sequence corresponding to the accession number.

2.6 Reference gene selection

A selection of reference gene was done using 8 commonly used reference genes (*ACTB, HMBS, HPRT1, TBP, TOP2B, YWHAZ, RPL4,* and *PPIA*). Their expressions in two samples from both IUGR and NBW piglets at each time points were measured using RT-qPCR as described above. Primers for *ACTB, HMBS, HPRT1, TBP, TOP2B* and *YWHAZ* were obtained from Erkens et al. (Erkens *et al.*, 2006), while primers for *RPL4* and *PPIA* were designed using Primer3Plus as above mentioned. The raw data were analyzed using the geNorm algorithm (Vandesompele *et al.*, 2002). The stepwise exclusion of the reference gene with the least stable expression showed that TBP was the two most stably expressed reference genes in the analyzed samples (**Figure II–1**). Thus, TBP was used as the reference gene in this study to normalize the raw data of RT-qPCR.

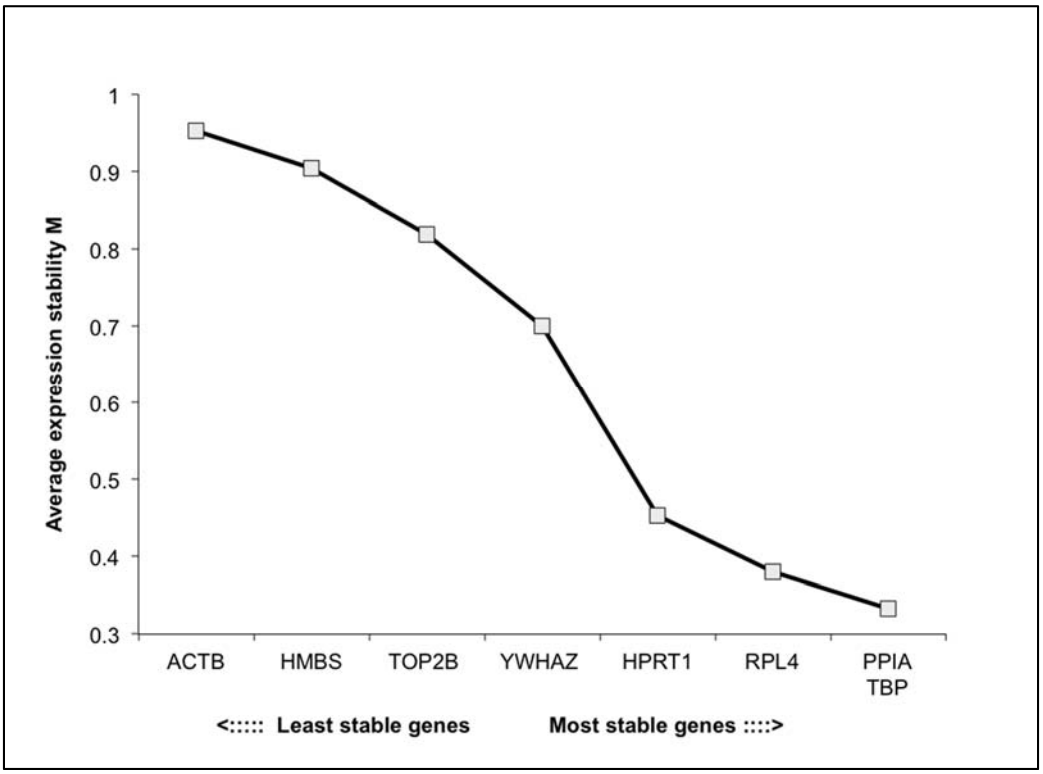

Figure II–1 *GeNorm result of reference gene stability.*

2.7 Gel electrophoresis and Western Blotting

To confirm whether the transcriptional regulation also occurred at the translational level, Western blotting analyses were applied. To this purpose, 100 mg of frozen samples of the proximal SI mucosa were homogenized in T-PER reagent (w/v, 1:10, Pierce Chemical, Rockford, IL, USA) supplemented with a complete protease inhibitor cocktail (Pierce Chemical, Rockford, IL, USA). The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL, USA). Afterwards, 30 µg of each protein extract was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini protean TGX 4% to 15% gradient gels (Bio-Rad Laboratories N.V., Winninglaan, Temse, Belgium). Separated proteins were transferred to a PVDF membrane (Merck KGaA, Darmstadt, Germany) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). After blocking for 1 h with 5% nonfat dry milk in phosphate buffered saline plus 0.1% Tween-20 (PBST), blots were probed overnight (4 °C) with one of the following primary antibodies: i) anti-occludin (Rabbit monoclonal antibody (mAb), 1:1000, Abcam, Cambridge, UK), ii) anti-HO (Rabbit mAb, 1:1000, Abcam, Cambridge, UK), iii) anti-GPX (Rabbit mAb, 1:1000, Cell signaling Technology, Danvers, MA, USA), iv) anti-TNFα (Rabbit mAb, 1:500, Cell Signaling Technology, Danvers, MA, USA), and v) anti-GAPDH (Rabbit mAb, 1:1000, Cell Signaling Technology, Danvers, MA, USA). The blots were washed in PBST and subsequently incubated for 30 min (room temperature) with HRP conjugated goat anti-rabbit IgG (1:1000, Pierce Chemical, Rockford, IL, USA). The protein bands were visualized using the enhanced chemiluminescence substrate (Bio-Rad Laboratories, Hercules, CA, USA) in combination with the Chemi Doc MP system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with Image Lab 4.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8 Statistical analysis

Normality of data and homogeneity of variance were tested using the Brown-Forsyth test in SAS Enterprise Guide 7 (SAS Institute, Cary, NC, USA). Data were analyzed by paired T-test comparison between IUGR and NBW piglets, and one-way analysis of variance and orthogonal contrasts (Tukey's honestly significant difference) for the different time points (days postnatal) within each type of piglets; using animal as experimental unit (n = 6). Data are expressed as means and standard error of mean (SEM), and $P < 0.05$ was considered significant.

3 RESULTS

3.1 Body weight and average daily gain of piglets

Average body weight of IUGR and NBW piglets sampled at birth (DPN0) were 0.77 kg and 1.29 kg, respectively ($P < 0.01$; **Table II-2**). Between DPN3 and DPN19, IUGR piglets consistently showed lower body weights compared to their NBW littermates ($P < 0.01$). At the end of the trial (DPN19), the body weight of IUGR piglets was 60% lower ($P < 0.01$) than that of NBW piglets. The average daily gain of IUGR piglets during the first 19 days of suckling period was constantly lower than the NBW littermates ($P < 0.05$).

Table II–2 *Body weight of sampled IUGR and NBW piglets at birth and during the neonatal period* [#]

	DPN0	DPN3	DPN8	DPN19
Body Weight (kg)				
IUGR	0.77 ± 0.07**	1.09 ± 0.05**	1.69 ± 0.15**	3.01 ± 0.16**
NBW	1.29 ± 0.08	1.73 ± 0.03	2.88 ± 0.24	4.81 ± 0.32
Average daily gain (kg/d)	**DPN0-3**		**DPN0-8**	**DPN0-19**
IUGR	0.095 ± 0.027*		0.104 ± 0.016*	0.118 ± 0.009**
NBW	0.150 ± 0.015		0.178 ± 0.019	0.194 ± 0.012

[#] Values are means ± SEM, n = 6. *, $P < 0.05$ vs NBW group. **, $P < 0.01$ vs the NBW group.

3.2 Permeability measurements in proximal and distal SI

Compared with NBW piglets, IUGR piglets showed both higher FD4 and HRP fluxes in the proximal SI at DPN0 and DPN3 ($P < 0.01$), while at DPN8 and DPN19, neither FD4 nor HRP flux was different ($P > 0.05$) between IUGR and NBW piglets (**Fig. II–2**). Both FD4 and HRP fluxes remained at the same level of DPN0 in IUGR piglets during the whole neonatal period, while in NBW piglets, FD4 flux increased from DPN3 to DPN8 and to DPN19 ($P < 0.05$) and HRP flux increased on DPN19 in NBW piglets. As for distal SI, only HRP flux at DPN0 differed between the two types of piglets ($P < 0.01$).

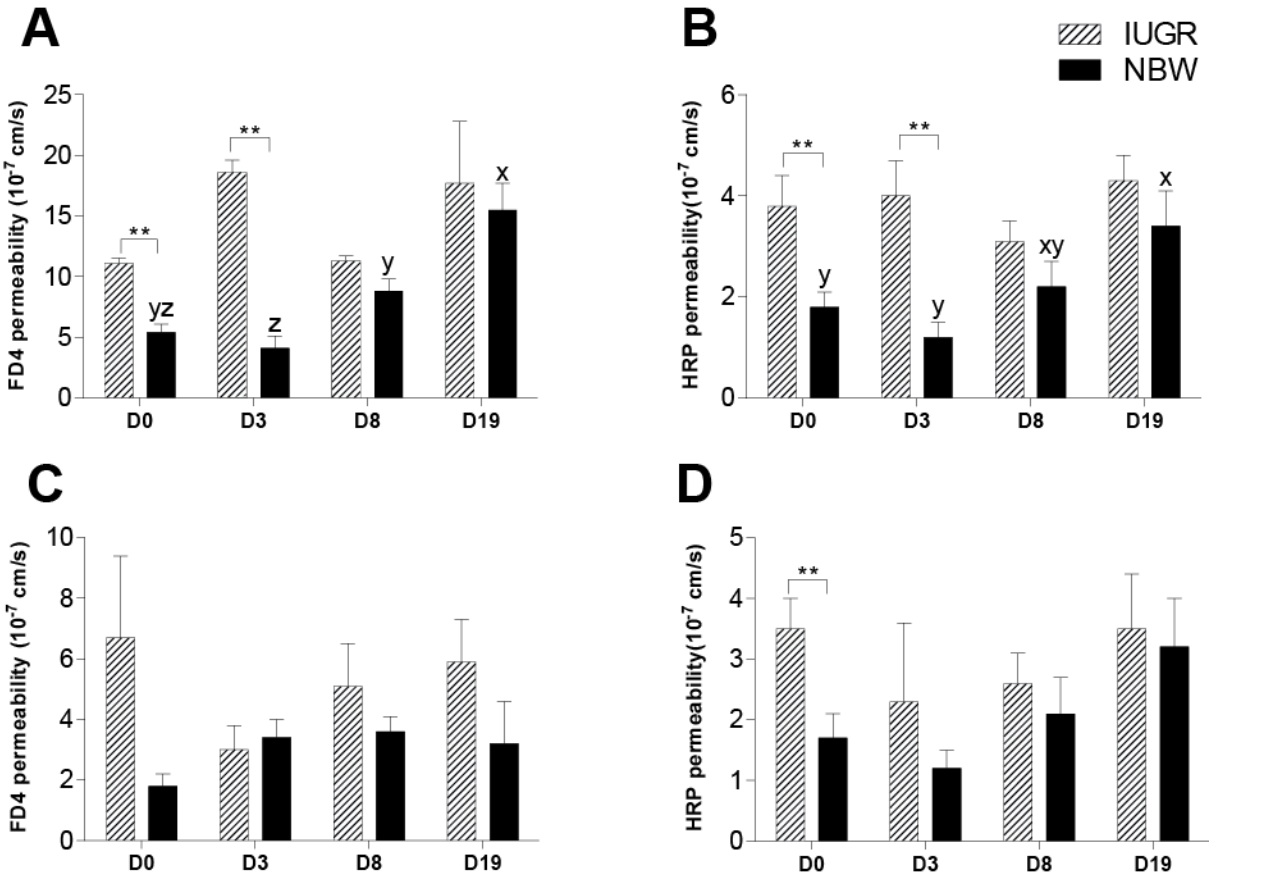

Figure II–2 *Intestinal permeability of IUGR and NBW piglets during the neonatal period. (A) FD4 flux at 5% of SI; (B) HRP flux at 5% of SI; (C) FD4 flux at 75% of SI; (D) HRP flux at 75% of SI. Data are means ± SEM; n = 6 for each group. **, P < 0.01 IUGR vs NBW. Means with different lower cases (x, y and z) represent differences between time points within IUGR piglets or NBW piglets, respectively (P < 0.05).*

3.3 Tight junction protein and TNF-α expressions in the proximal SI

The protein and gene expression of occludin (*OCLN*) and the gene expression of zonulin can be found in **Fig. II-3.** On DPN0 and DPN19, the expression of *OCLN*, both on translational and transcriptional level, was different between IUGR and NBW piglets ($P < 0.01$). Meanwhile, *OCLN* expression was upregulated on DPN3 in both types of piglets ($P < 0.01$). However, on DPN19, *OCLN* expression was downregulated in IUGR piglets while no difference was observed in NBW piglets ($P > 0.05$). Zonulin was not affected by type of piglets, neither by days postnatal ($P > 0.05$). *TNF-α* was lower in IUGR compared to NBW at DPN0, whereas it was higher at DPN19 ($P < 0.01$) (**Fig. II-3B**). Additionally, *TNF-α* mRNA level did not change in NBW subjects throughout the experimental period postnatal ($P > 0.05$), while it experienced twice upregulation in the IUGR population, respectively on DPN3 and DPN19 ($P < 0.01$). Eventually, from DPN0 to DPN3, the expression of *TNF-α* increased more than 4-fold ($P < 0.01$).

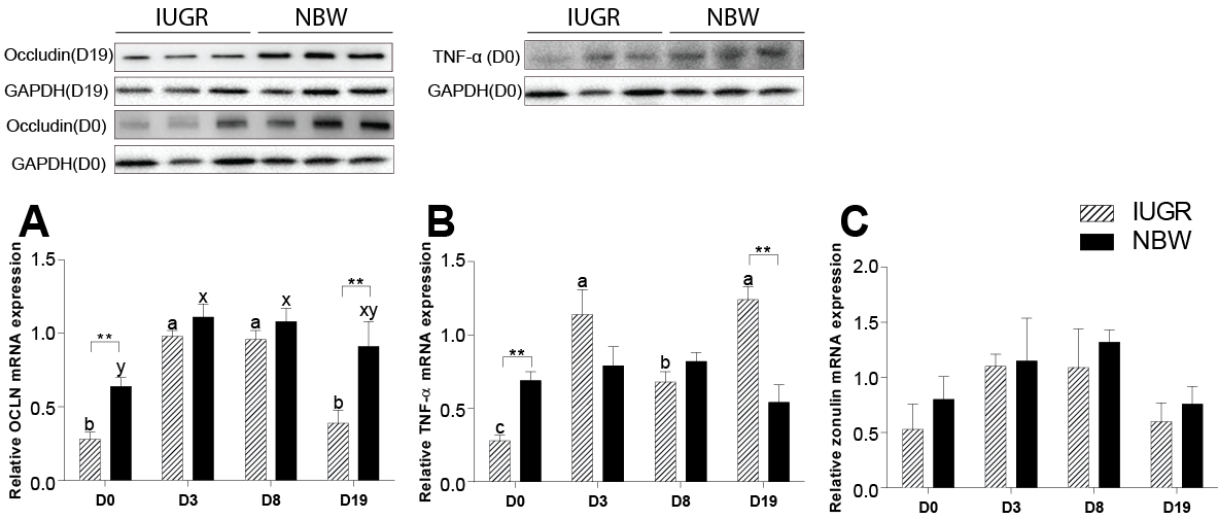

Figure II–3 *Relative expressions of OCLN (A), TNF-α (B) and zonulin (C) with the corresponding western blot result at 5% of SI from IUGR and NBW neonates at DPN0, DPN3, DPN8 and DPN19. Data are means ± SEM; n = 6 for each group. **, P < 0.01 IUGR vs. NBW. Means with different letters represent difference between different time points within IUGR piglets (a, b and c) or NBW piglets (x, y and z), respectively.*

3.4 GSSG/GSH ratio and MDA content in the SI.

At all the time points in this study, GSH, GSSG, GSSG/GSH ratio and MDA content did not differ neither in proximal nor distal SI between the two types of piglets ($P > 0.05$). As an example, data for the proximal SI are given in **Fig. II-4**. However, in the proximal SI of IUGR piglets, the GSH content showed first an increase on DPN3, then a drop on DPN19 ($P < 0.05$), while for NBW piglets the GSH content remained high and declined only on DPN19. In addition, the increase in GSSG/GSH ratio between DPN0 and DPN3 was limited for NBW ($P > 0.05$); whereas a nearly 2-fold increase was seen for IUGR but not statistically significant ($P > 0.05$). MDA concentration in the proximal SI was significantly higher at DPN0 compared to other sampling points, both in IUGR and NBW piglets ($P < 0.05$).

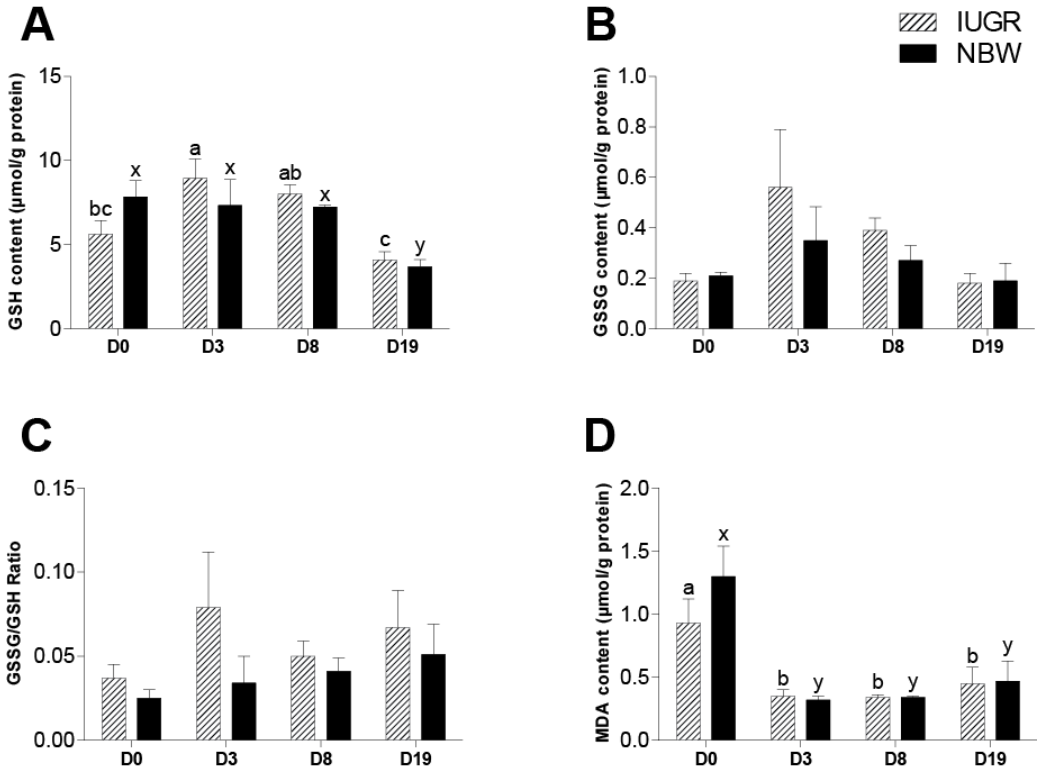

Figure II–4 *GSH content (A), GSSG content (B), GSSG/GSH ratio (C) and MDA content (D) at 5% of SI at DPN0, DPN3, DPN8 and DPN19. Data are means ± SEM; n = 6 for each group. In all cases, IUGR vs. NBW, P > 0.05. Means with different letters represent difference between different time points within IUGR piglets (a, b and c) or NBW piglets (x, y and z), respectively.*

3.5 Antioxidant capacity in the proximal SI mucosa

There was no difference of *SOD1* mRNA level between IUGR and NBW piglets during DPN0 to DPN19 (**Fig. II-5**). On DPN0, compared with NBW piglets, lower expression of heme oxygenase 1 (*HMOX1*), *CAT*, peroxisome proliferator-activated receptor gamma (*PPARG*) and thioredoxin reductase 1 (*TXNRD1*) was found in the IUGR subjects ($P < 0.01$). *GPX1* and Vanin-1 (*VNN1*) showed no difference between IUGR and NBW piglets on DPN0 ($P > 0.05$). Also, on DPN3 and DPN8, all the measured redox sensitive genes showed no difference between IUGR and NBW piglets ($P > 0.05$). On DPN19, the mRNA level of *HMOX1*, *CAT*, *PPARG*, *GPX1* and *TXNRD1*, was lower in IUGR piglets than that in NBW piglets ($P < 0.01$). In contrast, IUGR piglets showed higher *VNN1* expression than NBW piglets on DPN19 ($P < 0.01$). Interestingly, in IUGR piglets, all of the measured genes experienced an upregulation on DPN3, followed by a downregulation towards DPN19 ($P < 0.05$), except *VNN1*, which showed only an upregulation on DPN19 and *GPX1*, which exhibited a downregulation on DPN19 ($P < 0.05$). In NBW piglets, *GPX1* and *VNN1* were found being down regulated when DPN19 was compared with DPN0 ($P < 0.05$), whereas *SOD1* was upregulated in that respect ($P < 0.05$).

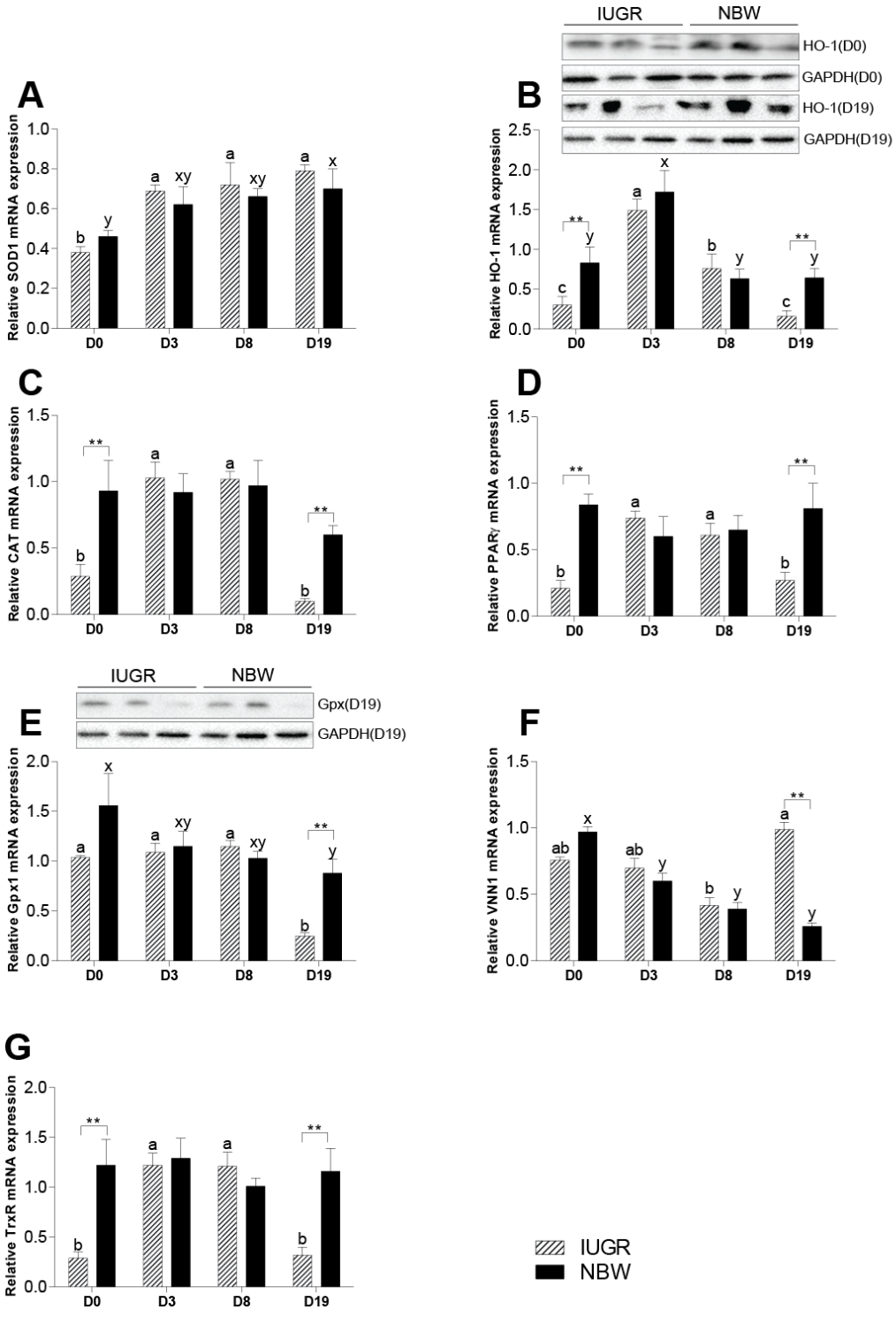

Figure II–5 *Relative mRNA level of SOD1 (A), HMOX1 (B), CAT (C), PPARG(D), GPX1 (E), VNN1 (F) and TXNRD1 (G) with the corresponding western blot results at 5% of SI from IUGR and NBW neonates at DPN0, DPN3, DPN8 and DPN19. Data are means ± SEM; n = 6 for each group. **P < 0.01 IUGR vs. NBW. Means with different letters represent difference between different time points within IUGR piglets (a, b and c) or NBW piglets (x, y and z), respectively.*

3.6 Histo-morphological and cell density measurements in the proximal SI.

Since major differences in intestinal permeability were found in the proximal SI at DPN0 and DPN3, histo-morphological and cell density measurements were done for these cases (**Table II-3**). Results show that there was no difference between IUGR and NBW piglets on DPN0 and DPN3 for villus height, villus surface area and crypt depth. A tendency of higher cell density in the lamina propria was observed in IUGR piglets on DPN0 ($P = 0.06$).

Table II–3 *Histo-morphological and cell density measurements in the proximal SI from piglets on DPN0 and DPN3* *

	DPN0		DPN3	
	IUGR	NBW	IUGR	NBW
Villus height (μm)	672 ± 35	671 ± 43	730 ± 77	752 ± 38
Villus surface area (10^{-3} cm^2)	1.09 ± 0.05	1.18 ± 0.13	1.35 ± 0.10	1.45 ± 0.06
Crypt depth (μm)	93 ± 6	99 ± 7	112 ± 2	116 ± 4
Lamina propria cells (%)	65 ± 6[#]	51 ± 4	67 ± 2	69 ± 3

*Values are means ± SEM, n = 6 per group. There was no difference between IUGR and NBW piglets on DPN0 and DPN3 for villus height, villus surface area and crypt depth.
[#]A tendency of higher percentage of lamina propria cells in IUGR piglets on DPN0 ($P = 0.06$).

4 DISCUSSION

Using the neonatal piglet model, we demonstrated that IUGR is associated with major changes in the epithelial barrier function, as well as antioxidant capacity in the proximal SI mucosa. Also, IUGR modified the TNF-α cytokine expression on the first day of life and at the end of the neonatal period. However, the redox status represented by the GSSG/GSH ratio and MDA concentration, and intesinal absorptive area were not affected by IUGR during the neonatal period.

A disturbed barrier function is associated with increased permeation of macromolecules through paracellular or transcellular pathways. Previously, a disruption in colonic barrier was found in neonatal

IUGR rat (Fanca-Berthon *et al.*, 2009). The two markers FD4 and HRP, used in our study, are primarily used to study the permeation through paracellular and paracellular/transcellular pathways, respectively (Boudry, 2005). The neonatal intestinal epithelium of pigs is characterized by a high permeability to macromolecules after birth, allowing the absorption of maternal immunoglobulins by receptor-mediated endocytosis, providing the pig passive immunity (Westrom *et al.*, 1984; Pacha, 2000). This high permeability gradually decreases after birth, which is called "gut closure". The process of gut closure is commonly completed before 36 h after birth. In our study, the DPN0 sampling time was > 12 h after birth and every piglet had prior access to colostrum. Since gut closure is related to colostrum intake (Westrom *et al.*, 1984), and it is well known that the colostrum intake of low-birth-weight piglets is delayed and inadequate (Le Dividich *et al.*, 2005; Quesnel *et al.*, 2012; Yuan *et al.*, 2015), we can assume a delayed gut closure in the IUGR piglets. This might explain the higher HRP flux in the IUGR compared to the NBW piglets at DPN0. However, FD4 represents the paracellular permeability and should not be affected by the gut closure phenomenon. Therefore, we assume that the higher permeability in the IUGR neonates truly indicates that the barrier function is compromised, which can promote the translocation of antigens and/or bacteria causing inflammatory responses. Similarly, D'Inca et al. speculated the IUGR induced elevated expression of peptide transporter PEPT1 and high density of adherent bacteria and translocation which may result in a decrease in barrier function (D'Inca *et al.*, 2011). In terms of ontogenetic maturation, increased permeability was found in NBW piglets at DPN19 as compared to prior sampling dates, indicating that at the end of the neonatal period (3-week weaning system) a higher paracellular permeability is present. A similar observation was reported by Huygelen *et al.* (2014), who found a higher lactose/mannitol ratio in both IUGR and NBW piglets at the end of neonatal period . In the present study, greater differences in permeability were found in the proximal SI compared to distal SI, in line with other parameters indicating that the development of the intestinal epithelium is site specific (Jolma *et al.*, 1980;

Drozdowski *et al.*, 2010; Yuan *et al.*, 2015). These phenomena might be related to differences in local or maternally derived growth factors and/or pancreatic secretion to the gut lumen (Telemo *et al.*, 1987; Martin *et al.*, 1993, 1997).

Zonulin is the only physiological modulator of intercellular tight junctions described so far that is involved in trafficking of macromolecules (Fasano, 2011). Since no difference was observed in the proximal SI villus area and expression of zonulin at DPN0 and DPN3 between IUGR and NBW, we postulate that the absorptive area of the proximal SI was not responsible for the differences found in the *ex vivo* permeability assay in these early days of neonatal period. This is in contrast with the previous studies showing that the villus area and villus height of ileum of 2-day old IUGR piglets were lower than the NBW ones (D'Inca *et al.*, 2010b), suggesting that the absorptive area difference is intestinal site-specific. The rapid increase of *TNF-α* mRNA in the proximal SI of IUGR piglets during the first 3 days of life, is consistent with earlier results regarding other proinflammatory cytokines, like interleukin-6 (D'Inca *et al.*, 2011). In line with this, a tendency of higher cell density in the lamina propria was observed in IUGR piglets, however only on DPN0. Thus, the greater capacity for epithelial permeation during the first days of life in IUGR piglets may induce a high load of antigens, which subsequently triggers the intestinal defense system. As HRP and FD4 can both be transported through the paracellular pathway, we can assume that the tight junction sealing the space between adjacent cells may be affected by IUGR. OCLN is a unique marker of tight junction integrity found in epithelial barriers and its presence or relative absence could perhaps shed a light on the type of permeability that is affected (Blasig *et al.*, 2011). Many reports have described that the specific membrane localization and regulatory contribution to barrier tightness of OCLN can be down regulated by oxidants and prevented by antioxidants (Walter *et al.*, 2009; Blasig *et al.*, 2011). The higher tight junction permeability in IUGR piglets on DPN0 could be a result of lower *OCLN* expression. Although on DPN3, *OCLN* mRNA level showed no difference between IUGR and NBW piglets, we should bear

in mind that the regulation may happen on other tight junction proteins or their association with the actin cytoskeleton.

Shifts in the GSSG/GSH redox potential due to mucosal GSH oxidation to GSSG would occur during acute or chronic intestinal oxidative stress (Shan *et al.*, 1990). A metabolome study reported a decreased GSH concentration in 21-d old IUGR compared to NBW piglets (He *et al.*, 2011). In our study, no significant difference was found for GSH, GSSG, and GSSG/GSH ratio between IUGR and NBW piglets on each separate time point. However, in IUGR subjects, GSH markedly increased from DPN0 to DPN3, indicating a different regulation on GSH production or consumption in proximal small intestinal mucosa. MDA is one of the toxic metabolites produced by lipid peroxidation (Zhang *et al.*, 2014), it is commonly used as biomarker for oxidative stress (Nielsen *et al.*, 1997), but here its levels at different days in neonatal period were not affected by type of piglet. Only from DPN0 to DPN3, intestine mucosal MDA both decreased in IUGR and NBW piglets, suggesting IUGR and NBW subjects may have similar redox status in the proximal SI during the neonatal period.

On the other hand, birth oxidative stress could induce excessive ROS production, which is known to induce upregulation of the expression of antioxidant enzymes in SI of neonates (Yin *et al.*, 2013). Although no difference was found in the redox status represented by GSSG/GSH ratio and MDA, remarkable differences both at the translational and transcriptional level of redox-sensitive proteins were found. The gene expressions of antioxidant enzymes *HMOX1, CAT,* TXNRD1 as well as nuclear factor *PPARG* in the proximal SI all showed downregulation on DPN0 and DPN19 in IUGR compared to NBW piglets in this study. It is unclear why no differences in gene expression were observed at the intermediate sampling points, but only on DPN0 and DPN19. Possible reason for the age-specific differences between IUGR and NBW piglets could be the intake of colostrum and milk. Within the first few hours after birth, completion for mammary glands occurs among newborn piglets, the anterior and middle mammary gland are preferred(Kim *et al.*, 2000). IUGR piglets refer to the

runts in a litter, so they may have less chance to suckle from anterior mammary gland. It has been proved that the Igs and lactoferrin concentration are different in colostrum/milk from anterior and posterior mammary gland(Wu *et al.*, 2010b). Thus, the different colostrum/milk source (anterior/posterior mammary gland) could also be a reason these differences. Wang *et al.* (2010) found a reduction of protein disulfide isomerase-associated 3 expression in IUGR subjects, and suggested that in IUGR redox-sensitive signaling pathways are attenuated. In line with a previous study on NBW piglets, *SOD1* expression showed an upregulation during the whole neonatal period in piglets (Yin *et al.*, 2013). SOD is the soluble Cu/Zn SOD localized in the intermembrane space or cytosol (Tsang *et al.*, 2014), converting superoxide into hydrogen peroxide (H_2O_2) and molecular oxygen. Inhibition of *SOD1* induced both pro-oxidant effects mediated by an excess of superoxide, and antioxidant effects caused by a decrease in H_2O_2 (Juarez *et al.*, 2008). In our study, an imbalance between H_2O_2 production and scavenging, representing by similar mRNA level of *SOD1*, and lower mRNA level of *GPX1* or *CAT* in IUGR piglets, compared to NBW littermates, was noticed on DPN0 and in particular on DPN19. Accumulation of H_2O_2 resulting from this imbalance, could either increase oxidative damage or activate other antioxidant systems. Also, in a previous study with weaned piglets, we demonstrated that GPX activity was lower in IUGR piglets compared to NBW littermates (Michiels *et al.*, 2013), thus corroborating with the current findings on mRNA level. Furthermore, *TRNXD1* mRNA level was also found to be lower in IUGR piglets. TXNRD1 and glutaredoxin 2 are the only enzymes known to keep thioredoxin in the reduced state, during which process ROS are scavenged (Zhang *et al.*, 2014). Together with GSSG/GSH, the thioredoxin/thioredoxin disulfide (Trx/TrxSS) couple comprises the major intracellular redox system in gut epithelial cells (Circu & Aw, 2012). Hence, the lower expression of *TRNXD1* may also reflect a disturbed antioxidant capacity of IUGR intestinal mucosa. HO catalyzes the degradation of heme and generates CO, biliverdin and iron (Tenhunen *et al.*, 1968). Enhanced *HMOX1* expression could be induced by oxidative stress and has

indirect cytoprotective responses against oxidative stress (Vile *et al.*, 1994; Otterbein & Choi, 2000). It has been shown HO is crucial in alleviating oxidative stress in intestinal epithelial cells (Oates & West, 2006). We saw that *HMOX1* expression in the IUGR pigs was reduced on DPN0 and DPN19, giving further proof for altered antioxidant protection in case of an IUGR. PPARγ is a member of the nuclear hormone receptor superfamily of transcription factors that regulates genes involved in lipid and glucose metabolism. Mounting reports have indicated that PPARγ regulates oxidative status and inflammation (Blanquicett *et al.*, 2010). VNN1 is an ectoenzyme anchored at the surface of epithelial cells that provides cysteamine to tissues (Berruyer *et al.*, 2006). In *VNN1* knock-out mice, the lack of cysteamine is associated with elevated endogenous GSH stores in the tissues, leading to a better resistance to oxidative stress exposure (Berruyer *et al.*, 2004; Martin *et al.*, 2004). Previous studies have also shown that VNN1 is an oxidative stress sensor and acts as a pro-inflammatory molecule by inhibiting PPARγ in epithelial cells (Blanquicett *et al.*, 2010; Zhang *et al.*, 2011). Accordingly, the increased *VNN1* mRNA level and decreased *PPARG* mRNA level in the proximal SI of IUGR piglets at DPN0 and DPN19 from this study provide another line of evidence for the presence of disturbed redox-signaling in IUGR offspring during postnatal life. Taken together, although the redox-sensitive proteins investigated in this study are regulated through different pathways, they all show lower expression in the proximal SI from IUGR piglets, suggesting that there might be a delay in development of the antioxidant system in the intestine of IUGR subjects. Meanwhile, we cannot exclude that differences in gene expression are associated with differences in proliferation and turnover of epithelial cells during the early life of neonates. The exact cell types involved in this differential gene expression warrants further investigation.

5 CONCLUSION

In summary, results of this study provide evidence for a transient alteration of tight junction permeability of proximal small intestinal mucosa, associated with a reduced expression of the tight

junction protein *OCLN* in IUGR neonates. In addition, although redox status represented through GSSG/GSH ratio and MDA was not affected by IUGR, there might be a delay in the activation and response of the antioxidant system in the proximal SI of IUGR neonatal piglets. Also, the regulation mechanism on transcriptional level of redox-sensitive genes might be independent from the redox regulator GSSG/GSH couple. Collectively, these changes may explain some mechanisms responsible for intestinal dysfunction in IUGR neonates. The consequences and signaling pathways of these modifications observed in IUGR neonates for the antioxidant system development remain to be elucidated.

Chapter III

Effect of intrauterine growth restriction on the intestinal barrier function, redox status and redox-sensitive genes expression during the postweaning period

ABSTRACT

Rare information can be found about the small intestinal development of intrauterine growth restriction (IUGR) piglets after weaning. Therefore, the effect of IUGR on small intestinal (SI) permeability, redox status and redox-sensitive gene expressions were investigated in piglets from weaning to day 28 postweaning. Thirty pairs of IUGR and normal birth weight (NBW) sex-matched littermates were marked at birth and weaned at day 19 of age. Small intestinal mucosae were obtained at 0, 2, 5, 12 and 28 days postweaning (DPW0, DPW2, DPW5, DPW12 and DPW28). The intestinal epithelial permeability was assessed ex vivo by 4 kDa fluorescein isothiocyanate-dextran (FD4) and horseradish peroxidase (HRP) fluxes across the epithelium, while intestinal architecture was assessed by histomorphological measurements. The redox status of glutathione disulfide/glutathione was determined, while transcriptional expressions of redox-sensitive proteins were quantified. Compared to the NBW ones, weaned piglets born with IUGR exhibited higher HRP fluxes on DPW5, but lower HRP fluxes on DPW12, both in the proximal small intestine (SI, all $P < 0.05$). Besides, on DPW5, IUGR piglets showed higher proximal SI villus height/crypt depth ratios and VNN1 mRNA level, but lower mRNA levels of *TNF-α, GPX1, PPARG and TXNRD1* (all $P < 0.05$). It appears that the intestinal maturation process and response to weaning is different in IUGR piglets compared to NBW ones. Collectively, results from this study add to our understanding the effect of IUGR on the intestine during postweaning period.

1 INTRODUCTION

Intrauterine growth restriction (IUGR) results in fetuses/neonates that do not reach their growth potential for a given gestational age. Pigs exhibit the most severe and naturally occurring IUGR among domestic animals, probably due to the imbalance between genetic selection for large litter size and the limited uterine capacity of the sow (Wu *et al.*, 2006). Since IUGR can lead to high perinatal mortality and morbidity, as well as reduced growth performance, it is a major concern in pig production (Quiniou *et al.*, 2002; Wu *et al.*, 2004a). Being born with IUGR has also been associated with continuous impaired intestinal development during the neonatal period (Wang *et al.*, 2010; Huygelen *et al.*, 2012; Huygelen *et al.*, 2014). Importantly, IUGR piglets have been shown to suffer from impaired antioxidant capacity, especially in the proximal small intestinal mucosa, evidenced by downregulated mRNA levels of antioxidant enzymes, such as glutathione peroxidase 1 *(GPX1), catalase* (*CAT),* heme oxygenase 1 (*HMOX1*) and *thioredoxin reductase* 1 (*TXNRD1*) on 19-d of age in a 21-d weaning system (Wang *et al.*, 2016). This could imply that IUGR can exert long-term influences on the postweaning intestinal antioxidant response of piglets.

Natural weaning is a gradual process. In contrast, in commercial settings weaning is an abrupt process, whereby the piglets are removed from the sow and are subjected to a myriad of stressors (Lalles *et al.*, 2004; Lalles *et al.*, 2007). Of particular interest, the transition from milk to a solid diet is accompanied with multiple changes in the SI development and functioning (Pluske *et al.*, 1997; Bauer *et al.*, 2011; He *et al.*, 2011). The disturbed intestinal barrier function induced by weaning has been associated with increased susceptibility to infections and piglet oedema disease (Wijtten *et al.*, 2011). In addition to the extensively documented weaning induced intestinal dysfunction and morphological alterations, it has been reported recently that weaning also causes disrupted oxidative status and inhibition of intestinal antioxidant systems (Wang *et al.*, 2008b). The mRNA levels of *GPX1* and *CAT* were lower in jejunum of 24-d-old piglets weaned on 21d of age, when compared to age-matched suckling piglets

(Wang *et al.*, 2008b; Zhu *et al.*, 2012). In contrast, Degroote *et al.* (2012; 2015) showed that GPX activity at 5 days postweaning (DPW5) is upregulated both in the proximal and distal SI mucosa when comparing to their level at weaning age. Though these literature data seem inconsistent, they suggest that weaning can affect the intestinal antioxidant response in piglets. Additionally, Yin *et al.* (2014) stated that on DPW3, plasma superoxide dismutase (SOD) and GPX activity was suppressed in a 14-d weaning system, compared to pre-weaning values .

Our previous work has shown that in low-birth-weight piglets, the development of the digestive tract in the postweaning period is delayed in the distal jejunum, and the plasma antioxidant capacity was lower when compared to age-matched normal birth weight (NBW) ones (Michiels *et al.*, 2013). Also, Zhang *et al.* (2015) reported that IUGR piglets showed reduced antioxidant enzyme activity, like Cu/Zn SOD (SOD1) in liver, and increased malondialdehyde (MDA) concentration in plasma on DPW14 in a 21-d weaning system, compared to the age-matched NBW littermates . To our knowledge, most of the differences at the level of small intestine (SI) between IUGR and NBW piglets were investigated during the neonatal period. Fragmentary information is available about the intestinal redox status and antioxidant system of IUGR piglets after weaning. Thus, we hypothesized that IUGR will affect the postweaning mucosal barrier function, morphology and antioxidant response in SI. This hypothesis was tested on 19-d old weaned piglets, by evaluating the intestinal histo-morphology, the *ex vivo* permeability of intestinal mucosa, as well as the gene expression of eight redox-sensitive biomarkers and inflammatory factors at different time points (DPW0, DPW2, DPW5, DPW12 and DPW28).

2 MATERIALS AND METHODS

2.1 Animal selection and tissue collection

The experiment was carried out according to the guidelines of the Ethical Committee of Ghent University (Belgium) for the humane care and use of animals in research (Nr. EC2011/195). During

two consecutive farrowing rounds, 27 sows with 14 or more live-born piglets (Topigs hybrid x Piétrain) was selected, and full term newborns were weighed within 12 hours after parturition to determine birth weight and gender. Thirty pairs of piglets (30 males and 30 females) were tagged with a unique ear tag. Birth weight was used as criterion to identify IUGR and NBW littermates. An IUGR piglet was defined as having a birth weight between 0.75 and 0.95 kg and belonging to the lower quartile of the litter birth weights, while a NBW littermate had a birth weight within ± 1 standard deviation of the mean birth weight of the whole litter. In total, sixty full term newborn piglets were selected. Cross-fostered piglets were excluded from the experiment. Average birth weight for all IUGR and NBW piglets in the study was 0.86 ± 0.02 and 1.30 ± 0.03 kg, respectively. All piglets were able to suckle the sow until weaning. After being weaned on 19-d of age, piglets were transferred to the nursing unit. Fifteen pens (2 × 2.2 ×0.8 m) were used, and 4 IUGR piglets or 4 NBW piglets were housed together in each pen. A cereal-soybean based diet (crude protein:17.3%, net energy: 2342 kcal/kg, **Table III-1**) was provided to the piglets and feed intake was recorded daily per pen. All the selected animals did not receive any antibiotic treatment prior or during the experiment, and no diarrhea was observed during the experimental period.

Table III–1 Composition of the weaner diet used for piglets from weaning till DPW28

Ingredients (%)	
Barley	33.00
Wheat	19.87
Soybean meal	9.00
Toasted soybean	12.00
Corn	5.00
Acid casein whey powder	4.69
Sugar beet pulp	2.00
Wheat gluten meal	3.83
Lactose	2.00
Lactic Acid	0.60
Monocalcium Phosphate	0.64
Calcium formate	0.20
Sodium formate	0.23
Potato protein	1.71
Limestone	0.68
Trace mineral and vitamin Premix[1]	1.80
L-lysine HCl	0.54
DL-methionine	0.21
L-threonine	0.15
L-valine	0.05
L-tryptophan	0.05
Soybean oil	2.22
Lard	1.00
Caculated Nutrient Values	
NEv [2] (1997) kcal/kg	2567
Crude protein (g/kg)	179
Crude fiber (g/kg)	83.7
Digestible lysine(g/kg)	11
Digestible methionine + cystine(g/kg)	6.6
Digestible threonine (g/kg)	6.7
Digestible tryptophan (g/kg)	2.2

[1] The mineral and vitamin premix provided the followed ingredients to per kg diet: Vitamin A, 15000 IU; Vitamin D_3, 2000 IU; Vitamin E, 100 IU; Vitamin K_3, 2.5 mg; Vitamin B_1, 0.8 mg; Vitamin B_2, 5.6 mg; Vitamin B_3, 12 mg; Vitamin B_6, 0.8 mg; Vitamin B_{12}, 0.026 mg; Niacine, 38 mg; Folic acid 0.4 mg; Fe^{2+}, 150 mg; Zn^{2+}, 115 mg; Cu^{2+}, 160 mg; Mn^{2+}, 34 mg; Se^{6+}, 0.25 mg; I^{-}, 1 mg; Propylgalate, 37.7 mg; BHT, 13.6 mg; Ethoxyquin, 1.92 mg; Phytase (EC3.1.3.26),540 FTU; Xylanse(EC 3.2.1.8), 70 AXC; Glucanase (EC3.2.1.6), 100AGL.

[2] Net energy for pigs CVB(1997), Centraal Veevoederbureau, Lelystad, The Netherlands.

At DPW0 (before removal from the sow), DPW2, DPW5, DPW12 and DPW28, six couples of IUGR and NBW gender-matched littermates (6 females and 6 males) were sampled. Piglets were first weighed, then killed by exsanguination following induction of terminal anesthesia by intra-peritoneal sodium pentobarbital (90 mg/kg BW). The SI, defined as the part of the gastro-intestinal tract between the pylorus and the ileo-cecal valve, was obtained and its length was measured. A 10 cm segment of proximal and distal SI (5% and 75% of total SI length, respectively) was taken for Ussing chamber measurements. Another 20 cm segment at these two sites were emptied and carefully flushed with saline. One part (5 cm) was immersed for 2h in 4% paraformaldehyde solution (0.1M, pH = 7.4) at room temperature for fixation, pending further processing for histo-morphology. The other part was placed on an ice-cold surface to obtain mucosa by scraping with a glass slide. Aliquots of mucosa were either used instantaneously for acid buffered aqueous extracts, or transferred to plastic 2 mL screw-capped cryovials, snap-frozen in liquid nitrogen and stored at -80°C pending gene expression analysis.

2.2 Histo-morphology

After fixation for 2h in 4% paraformaldehyde solution, intestinal sections were rinsed and kept in ice-cold phosphate buffered saline (pH = 7.4) and processed to paraffin blocks. Transverse paraffin sections (5 µm) were conventionally stained with haematoxylin-eosin (HE). Under magnification of 100 times, villus height and crypt depth were measured in 30 well-oriented villi and adjacent crypts for each tissue sample (Olympus BX 61, Analysis Pro; Olympus Belgium, Aartselaar, Belgium).

2.3 Biochemical assays

The acid mucosal extract was used for glutathione (GSH) and glutathione disulfide (GSSG) measurement. One gram of mucosa was homogenized in 10 mL ice-cold perchloric acid (10%) with a Braun homogenizer at 900 rpm, then centrifuged at 15,000 G for 15 min at 4°C. Subsequently, 0.5 mL of the resulting acid extract was transferred to tubes containing 50 µL of γ-Glu-Glu internal standard solution. The total protein content of mucosa was determined with the biuret method.

Mucosal GSH and GSSG were measured using a modified high performance liquid chromatography (HPLC) method (Reed *et al.*, 1980; Degroote *et al.*, 2012; Vergauwen *et al.*, 2015; Wang *et al.*, 2016) (see Chapter II).

2.4 *Ex vivo* measurement of intestinal permeability

Intestinal mucosal permeability was assessed *ex vivo* by measuring the translocation of fluorescein isothiocyanate dextran 4000 (FD4, 4 kDa) and horseradish peroxidase (HRP, 40 kDa) using the Ussing chamber technique as described in Chapter II. Appearance of FD4 and HRP in the serosal side was measured as previously described (see Chapter II).

The apparent permeation coefficient (Papp) was calculated as: Papp (cm/s): $(dc/dt) \times V/c_0/A$, whereby dc/dt is the change of serosal concentration in the 20- to 100-min period (cm/s); V is the volume of the chamber, c_0 is the initial marker concentration in the mucosal reservoirs and A is the area of the exposed intestine in the chambers (1.07 cm^2).

2.5 RNA isolation and reverse transcription real-time quantitative PCR (RT-qPCR)

Since main differences between IUGR and NBW piglets were found in proximal SI, the expression of redox-sensitive genes was investigated in the proximal SI mucosa samples only. The qPCR experiment was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline. Primers sequences used for the target and reference genes in the present study were obtained from our previous study (**Table II–1**), and only listed gene isoform can be specifically amplified. Mucosal total RNA was isolated using Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, CA, USA), including an on-column DNase treatment to remove genomic DNA (gDNA). The concentration (ranging between 250-1200 ng/µL) and purity (OD 260/280 ranging between 1.9-2.2) of RNA was measured with NanoDrop ND-1000 (Nanodrop Technologies, Thermo Scientific, Wilmington, DE, USA). Also, the quality of the extracted RNA was verified by identifying the 28S and 18S ribosomal RNA bands after being loaded

onto a 0.8% agarose gel, following RT-minus control to confirm the absence of any gDNA contamination. Then, 1 µg of high-quality gDNA-free RNA was converted to cDNA in the subsequent 20 µL RT reaction with ImProm-II cDNA synthesis kit (Promega, Madison, WI, USA), containing both oligo dT and random primers. The obtained cDNA was diluted 10 times with molecular grade water and verified by a RT step using 2 µL diluted cDNA.

The qPCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as previously describe in Chapter II. In this study, PCR efficiencies were consistently between 96%-102% with r^2 >0.996. The comparative quantification cycle (C_q) method were used to transform the C_q values to quantities, while the highest relative quantities for each target gene were set to 1, and PCR efficiency of every run were included to the normalization of raw target gene quantities. The relative mRNA level was expressed as a ratio of the target gene to the geometric mean of three most stable expressed reference genes (*TBP*, *HPRT1* and *PPIA*) selected by geNorm algorithm (Vandesompele *et al.*, 2002) using 8 commonly used reference genes (*ACTB, HMBS, HPRT1, TBP, TOP2B, YWHAZ, RPL4,* and *PPIA*), as previously describe in Chapter II.

2.6 Statistical analysis

Normality of data and homogeneity of variance were tested using the Brown-Forsyth test in SAS Enterprise Guide 7 (SAS Institute, Cary, NC, USA). Data were analyzed by paired T-test for the comparison between IUGR and NBW piglets on individual time points, and by one-way analysis of variance (Fisher's least significant-difference test, LSD) for testing the effect of day postweaning within each type of piglets, using animal as experimental unit (n = 6). Data are expressed as means and their standard errors (SEM), and $P < 0.05$ was considered significant.

3 RESULTS

3.1 Body weight, average daily gain, average daily intake & relative daily gain

During the 4-week postweaning period (DPW0-DPW28), IUGR piglets showed lower body weights

than their age-matched NBW ones on all the time points in this study (all P <0.05, **Table III–2**). Average daily gain was calculated based on the slaughtering weight and initial weight of the 6 piglets on difference sampling time point. During the first 2 days postweaning, IUGR piglets lost less weight than the NBW ones (P <0.05), but during the first 12 days postweaning, IUGR piglets showed less average daily gain (ADG) than the NBW littermates (P < 0.05). As for the average daily feed intake (ADFI), during the 28 days postweaning, IUGR piglets had significantly lower ADFI than the NBW ones (P < 0.05). Additionally, IUGR piglets showed higher relative ADG (calculated by the feed intake per kilo of mean body weight of the experimental period) than the NBW ones during the first 28 days postweaning (P <0.05).

Table III–2 *Body weight of sampled IUGR and NBW piglets on different days postweaning*

	Body weight on different days postweaning (kg)				
	DPW0	DPW2	DPW5	DPW12	DPW28
IUGR	4.41 ± 0.28^{b,*}	3.46 ± 0.27^{b,*}	3.80 ± 0.24^{b,*}	4.60 ± 0.52^{b,*}	11.01 ± 0.94^{a,*}
NBW	6.37 ± 0.32^{yz}	5.71 ± 0.24^{yz}	5.23 ± 0.36^{z}	7.87 ± 0.65^{y}	14.55 ± 0.96^{x}
	DPW0-2	DPW0-5	DPW0-12	DPW0-28	
	ADG (kg/d)				
IUGR	-0.098 ± 0.023*	-0.032 ± 0.015	0.055 ± 0.016*	0.25 ± 0.027	
NBW	-0.17 ± 0.015	-0.039 ± 0.012	0.12 ± 0.019	0.30 ± 0.028	
	ADFI (kg/d)				
IUGR	0.07 ± 0.026	0.09 ± 0.028	0.14 ± 0.018	0.36 ± 0.007*	
NBW	0.03 ± 0.002	0.14 ± 0.028	0.17 ± 0.015	0.41 ± 0.004	
	Relative ADG (%)				
IUGR	-2.73 ± 0.62	-0.76 ± 0.32	1.30 ± 0.29	6.41 ± 0.65*	
NBW	-2.82 ± 0.26	- 0.74 ± 0.22	1.88 ± 0.29	4.97 ± 0.16	

[1] Results are expressed as means ± SEM (n = 6). Different letters represent different means within either IUGR (a, b, c) or NBW (x, y, z) groups, and * IUGR is different from NBW piglets at the corresponding time point (P <0.05).
[2] Average feed intake data were based on pen level.
[3] Relative ADG was calculated by (Slaughtering weight – Weaning weight)/ Weaning weight.

3.2 *Ex vivo* intestinal permeability

Compared to age-matched NBW littermates, IUGR piglets showed significantly higher FD4 flux before weaning (DPW0, **Fig.III-1A**), higher HRP flux on DPW5, and lower HRP flux on DPW12 in the proximal SI (**Fig.III-1A**, all P <0.05). In the distal SI (**Fig.III-1C&D**), no differences in intestinal mucosal permeability between IUGR and NBW piglets on each time point were found (all P > 0.05).

In the proximal SI of IUGR piglets, comparing to the preweaning level, FD4 flux kept the preweaning value on DPW2 and subsequently dropped to about 80% of the preweaning value on DPW5, 12 and 28 (**Fig.III-1A**, P <0.05). Meanwhile, the proximal SI HRP flux was 3 times higher on DPW2 as compared to the preweaning level. After DPW2, the HRP flux decreased to the preweaning level (**Fig.III-1B**, P > 0.05). In NBW piglets, no difference with time were observed in FD4 flux in the proximal SI. The proximal HRP flux showed no difference with the preweaning level on DPW2 and DPW5, but it was 50% higher on DPW12 (**Fig.III-1D**, P < 0.05). In the distal SI, both IUGR and NBW piglets showed higher FD4 flux on DPW28 as compared to the preweaning level (**Fig.III-1C&D**, P < 0.05). In NBW piglets, the HRP flux (**Fig.III-1D**, P > 0.05) was 4 times lower than preweaning level on DPW2.

3.3 Intestinal histo-morphology

Results of histo-morphological measurements of intestinal sections of IUGR and NBW piglets at different time points postweaning are shown in **Table III–3**. Compared to the age-matched NBW ones, IUGR piglets showed higher proximal SI villus to crypt ratio (V/C ratio) on DPW5 (P <0.05). In the distal SI, no difference was found between IUGR and NBW piglets during the first 4-week postweaning (P >0.05 at all time points).

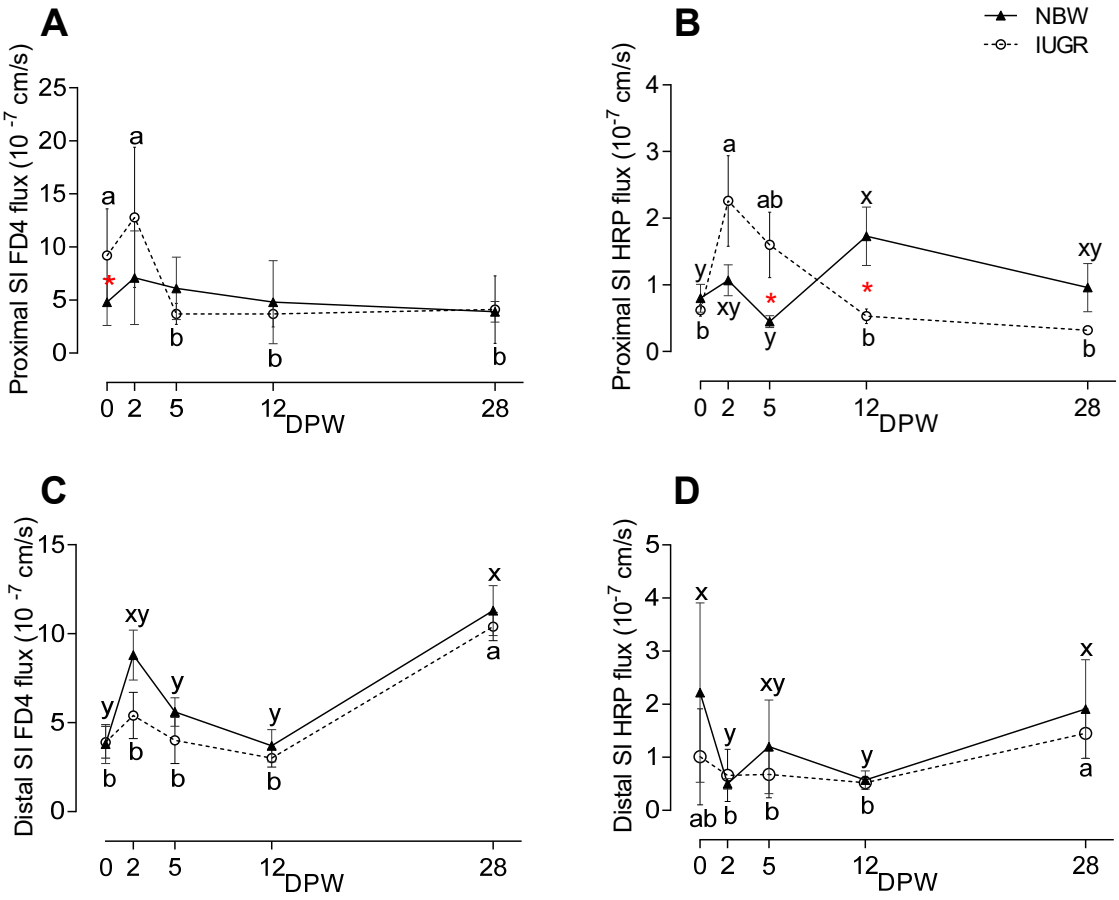

Figure III–1 *Ex vivo FD4 or HRP fluxes in proximal (A, B) and distal (C, D) small intestinal mucosa, respectively. Results are expressed as means ± SEM (n = 6). Different letters represent different means within either IUGR (a, b, c) or NBW (x, y, z) groups, and * represents differences between IUGR and NBW piglets at the corresponding time point (P < 0.05).*

In the proximal SI of IUGR piglets, villus heights and crypt depths were lower on DPW2, and crypts were deeper on DPW12 and DPW28, when compared to the preweaning level (P <0.05). In NBW piglets, compared to the preweaning level, the villus height in proximal SI was lower on DPW2 and DPW5, and crypt depth was higher on DPW5 and DPW12, (all P <0.05). As for the ratio of villus height to crypt depth, in the proximal SI, the lowest proximal V/C ratio was found on DPW12 in IUGR piglets, compared to the value on other time points (all P <0.05 except vs. DPW5), while in NBW piglets, the lowest V/C ratio was found on DPW5 in the proximal SI (all P < 0.05).

Compared to the preweaning level, villus height in the distal SI of IUGR piglets, continued to be lower at all time points after weaning, whereas crypt depth was only increased on DPW28, (all P <0.05). In NBW piglets, when compared to the preweaning level, villus heights remained lower from DPW5 (all P <0.05), but it was higher on DPW28 and DPW2 than that on DPW5 and DPW12 postweaning (all P < 0.05). The distal SI crypt depth was higher on DPW28, than other time points in NBW piglets (all P <0.05). Besides, compared to the preweaning level, the V/C ratio was lower on all the postweaning time points in IUGR piglets (P < 0.05), while in NBW piglets, V/C ratios were lower from DPW5 to DPW28, comparing to preweaning and DPW2 level (all P < 0.05).

Table III–2 *Effect of IUGR and days postweaning on intestinal morphology* [1]

		Days post weaning				
		DPW0	DPW2	DPW5	DPW12	DPW28
Proximal SI						
Villus height (µm)	IUGR	388 ± 30^{a}	302 ± 24^{b}	337 ± 22^{ab}	341 ± 32^{ab}	411 ± 14^{a}
	NBW	390 ± 34^{a}	285 ± 7.8^{b}	269 ± 35^{b}	398 ± 38^{a}	430 ± 34^{a}
Crypt depth (µm)	IUGR	161 ± 31^{b}	129 ± 7.2^{c}	171 ± 11^{ab}	204 ± 15^{a}	206 ± 14^{a}
	NBW	155 ± 17^{bc}	144 ± 12^{c}	196 ± 5.6^{a}	204 ± 10^{a}	183 ± 14^{ab}
V/C Ratio	IUGR	2.54 ± 0.12^{a}	2.34 ± 0.14^{ab}	2.09 ± 0.10^{bc,*}	1.87 ± 0.16^{c}	2.38 ± 0.03^{a}
	NBW	2.69 ± 0.19^{a}	2.05 ± 0.05^{b}	1.41 ± 0.20^{c}	2.18 ± 0.21^{ab}	2.63 ± 0.13^{a}
Distal SI						
Villus height (µm)	IUGR	546 ± 84^{a}	363 ± 24^{b}	211 ± 31^{c}	268 ± 31^{bc}	405 ± 37^{b}
	NBW	537 ± 76^{a}	410 ± 47^{ab}	244 ± 22^{c}	240 ± 30^{c}	357 ± 25^{bc}
Crypt depth (µm)	IUGR	91.2 ± 6.4^{bc}	80.8 ± 3.0^{bc}	77.8 ± 4.4^{c}	93.4 ± 5.5^{b}	126.6 ± 6.3^{a}
	NBW	95.9 ± 4.1^{b}	95.4 ± 4.2^{b}	90.8 ± 6.9^{b}	98.6 ± 6.2^{b}	119 ± 9.5^{a}
V/C Ratio	IUGR	5.79 ± 1.19^{a}	3.42 ± 0.39^{b}	1.25 ± 0.15^{c}	1.36 ± 0.12^{c}	2.37 ± 0.12^{b}
	NBW	5.01 ± 1.01^{a}	4.34 ± 0.75^{a}	1.36 ± 0.17^{b}	1.16 ± 0.12^{b}	2.22 ± 0.18^{b}

[1]Values are means ± SEM, n = 6 per group.

[a, b, c, d] Means in the same raw with different letters represent difference between different time points within in IUGR or NBW piglets (P <0.05).

[*] IUGR is different from NBW piglets on that time point postweaning (P < 0.05).

3.4 GSH, GSSG, GSSG/GSH ratio in the small intestinal mucosa

Compared to NBW piglets, in proximal intestine, IUGR piglets showed 50% higher GSH concentration before weaning (DPW0) (**Fig. III–2A**, P <0.05). In the distal SI, GSH concentration was higher in IUGR piglets on DPW5 and DPW28 postweaning (**Fig. III–2D**, P <0.05).

In the proximal SI, both IUGR and NBW piglets showed higher mucosal GSH concentration on DPW12 and DPW28 compared to the value on DPW0 and DPW2 (**Fig. III-2A**, P <0.05). In the distal SI of NBW piglets, the GSH concentration on DPW2 and DPW12 was significantly higher than the preweaning level and the level on DPW28 (**Fig.III-2D**, all P <0.05). The mucosal GSSG concentration in distal SI of either IUGR or NBW piglets was higher on DPW28 than the preweaning value and on DPW2 (**Fig.III-2E**, P <0.05). Regarding the GSSG/GSH ratio, in the proximal SI of both IUGR and NBW piglets, it was significantly lower on DPW2 than the preweaning level (**Fig.III-2C**, P <0.05).

3.5 Redox-sensitive and inflammatory factor genes expression in the proximal SI

Compared to NBW littermates, IUGR piglets showed downregulated *CAT* and *SOD1* expression (**Fig.III-3A&C**, P <0.05) before weaning. On DPW5, compared to the age-matched NBW piglets, lower mRNA level of *GPX1* (**Fig.III-3B**), *TNF-α* (**Fig.III-3D**), *TXNRD1* (Fig.III-3F), *PPARG* (Fig.III-3G), and higher mRNA level of *CAT* (Fig.III-3C) and *VNN1* (Fig.III-3H) were found in IUGR piglets (all P <0.05). Also, IUGR piglets showed a lower *TNF -α* mRNA level on DPW2, but higher *CAT* and *HMOX1* mRNA level on DPW28 as compared to the age-matched NBW ones (all P <0.05).

Compared to the preweaning level, in IUGR piglets, gene expressions of *SOD1, GPX1, TNF-α, TXNRD1* and *PPARG* were upregulated, whereas *VNN1* and *HMOX1* gene expressions were downregulated on DPW12 (P < 0.05). In NBW piglets, the expression of *GPX1, TNF, TXNRD1* and

PPARG were upregulated on DPW5, when compared with preweaning level, and the downregulation of *VNN1* and *CAT* were also observed on DPW5 (P <0.05).

4 DISCUSSION

It has been reported that IUGR piglets display attenuated antioxidant genes expression in the proximal SI at the end of neonatal period (19 or 21-d of age) (Wang *et al.*, 2010; Wang *et al.*, 2016). Additionally, several studies have suggested that weaned piglets born with IUGR showed lower circulatory antioxidant capacity as well as delayed development of the digestive tract (Michiels *et al.*, 2013; Zhang *et al.*, 2015). Weaning has been well documented for temporarily impairing intestinal barrier function and architecture (Pluske *et al.*, 1997; Moeser *et al.*, 2007a; Moeser *et al.*, 2007b; Moeser *et al.*, 2012). However, the intestinal permeability and redox status are poorly understood in weaned piglets born with IUGR. In the current study, we hypothesized that IUGR can affect the postweaning intestinal permeability and antioxidant responses in piglets. We investigated the dynamic changes in the permeability of intestinal mucosa, redox status as well as the gene expression of redox-sensitive biomarkers in weaned IUGR and NBW piglets. Results showed that in IUGR piglets the weaning stress induced distal SI barrier disorder is not observed. Also, it seems that the structural adaptation to weaning is depending on birth weight (IUGR vs. NBW).

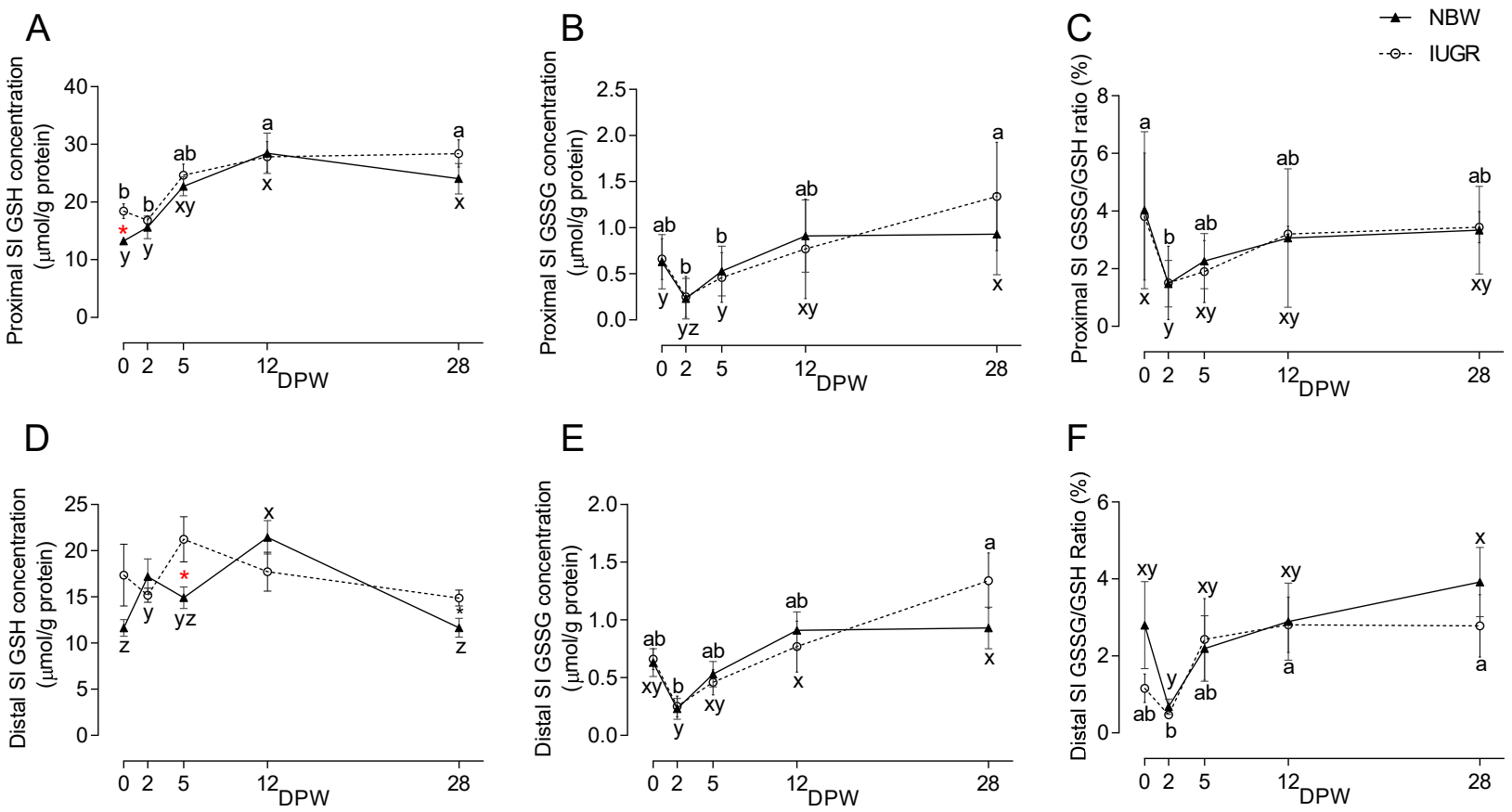

Figure III–2 GSH (A, D), GSSG (B, E) concentrations and GSSG/GSH ratio (C, F) in proximal or distal small intestinal mucosa, respectively. Results are expressed as means ± SEM (n = 6). Different letters represent different means within either IUGR (a, b, c) or NBW NBW (x, y, z) groups, and * represents differences between IUGR and NBW piglets at the corresponding time point (P<0.05).

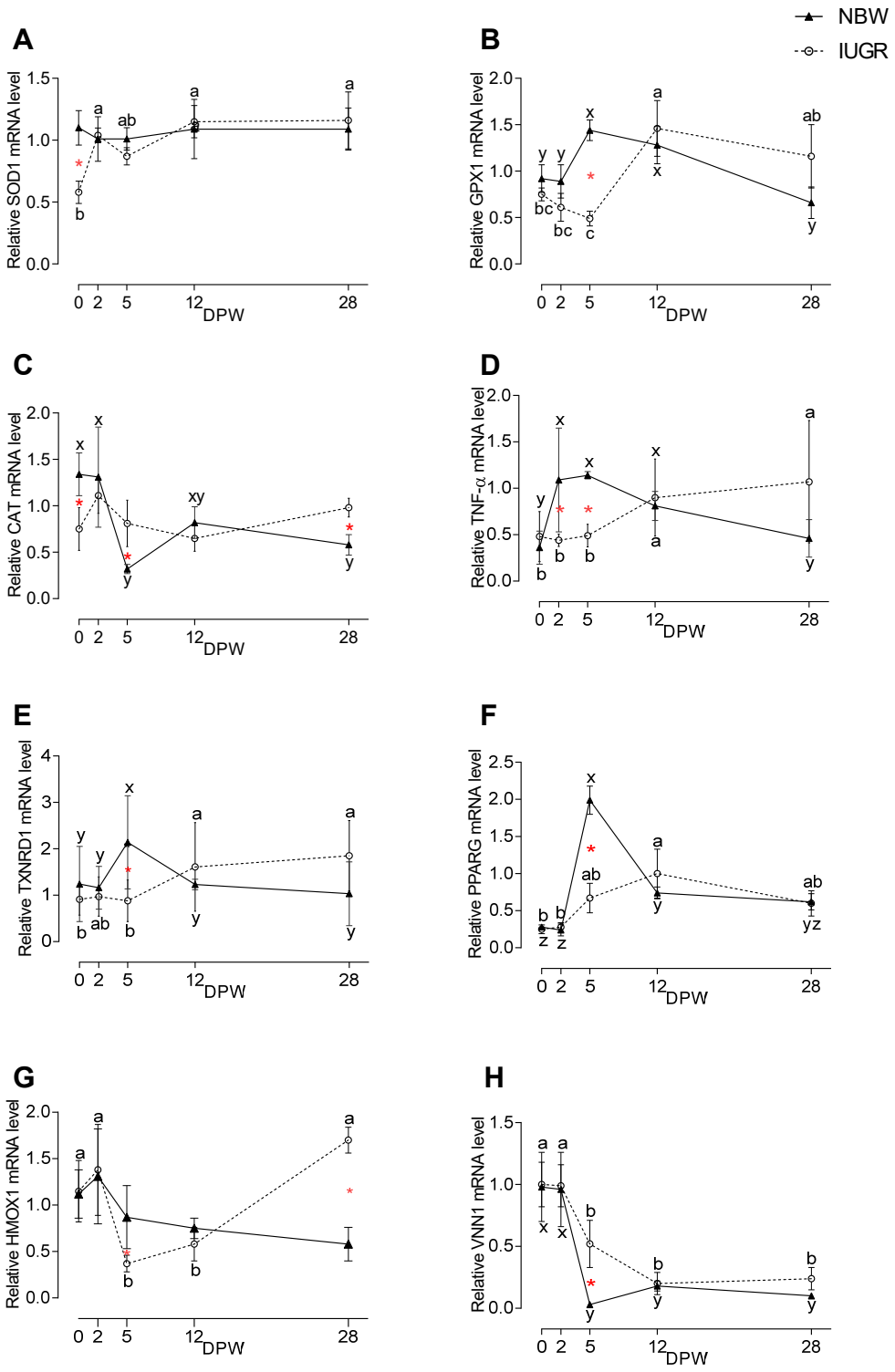

Figure III–3 *SOD1 (A), GPX1 (B), CAT (C), TNF-α (D), TXNRD1 (E), PPARG (F), HMOX1 (G) and VNN1 (H) gene expressions in proximal small intestinal mucosa of weaned IUGR and NBW age-matched littermates. Results are expressed as means ± SE (n = 6). Different letters represent different means within either IUGR (a, b, c) or NBW (x, y, z) groups, and * represents differences between IUGR and NBW piglets at the corresponding time point (P<0.05).*

4.1 IUGR affected weaning stress-induced intestinal permeability and histology alterations

HRP is a glycoprotein that has been widely used as a marker protein for studies of epithelial permeability (Heyman *et al.*, 1982; Heyman *et al.*, 1984; Heyman *et al.*, 1988). As a tracer macromolecule, the major transport route for HRP involves lysosomal degradation during the intracellular transport, which accounts for 88% of the transport. HRP that escapes metabolic degradation is transported by an alternative route requiring the structural and metabolic integrity of the epithelial cells (Heyman *et al.*, 1982). Since the HRP in this study was measured by enzymatic activity, it refers to the intact protein transport. Therefore, the higher HRP fluxes in the proximal SI of IUGR piglets on DPW5 as compared to the NBW littermates indicates that IUGR piglets have a more active endocytosis at this site. Similarly, Mickiwicz *et al.* (2012) reported increased uptake of macromolecules in jejunum of 28-d-old non-weaned IUGR piglets, likely due to the high presence of enterocyte lysosomes, which normally nearly disappear in the first two weeks after birth . Thus, our current finding of the higher transcytosis on DPW5 in IUGR piglets (24-day of age) could be attributed to a slower intestinal maturation and adaptation to postnatal life caused by IUGR. In contrast, Boudry *et al.* (2006) reported that jejunal HRP flux was not different in 28-d–old non-weaned IUGR piglets compared to the NBW ones , suggesting that the IUGR affected transcytosis might be intestinal site-specific and lysosomal vacuole-dependent. Notably, in the studies of Mickiwicz *et al.* (2012) and Boudry *et al.* (2006), the weaning process was not involved. Therefore, the effect of weaning cannot be exluded when comparing the results of current study with other literatures.

Stress is known to stimulate transepithelial macromolecular uptake while keeping the normal paracellular function (Saunders *et al.*, 1994; Kiliaan *et al.*, 1998; Gitter *et al.*, 2001; Ménard *et al.*, 2010). Thus, in IUGR piglets, the increased HRP fluxes on DPW2, as compared to the preweaning levels, with similar paracellular permeability are likely associated with weaning stress. In the proximal SI of NBW piglets, the enhanced transcytosis with unchanged paracellular permeability was observed

on DPW12, suggesting the weaning stress induced intestinal permeability alteration process in the proximal SI happens earlier in IUGR piglets. Also, the fact that HRP fluxes on DPW12 in IUGR piglets was lower compared with NBW ones, which may result from the enhanced transcytosis in NBW ones on DPW12, but attenuated transcytosis in IUGR ones, confirmed that the weaning stress induced increased transcytosis happens at different time points in IUGR and NBW piglets. These transcellular permeability shift could also be linked with the feed intake, during the first two days postweaning, the IUGR piglets showed two-fold feed intake than NBW piglets, indicating that certain amount of feed intake could stimulate the endocytosis process. Similar process was observed on DPW12 in NBW piglets, when the feed intake was recovered on DPW5.

In the distal SI, the temporary increase of paracellular permeability on DPW2 in NBW piglets was recognized as stress-induced intestinal barrier breakdown (Boudry *et al.*, 2004; Moeser *et al.*, 2007a; Montagne *et al.*, 2007; Hu *et al.*, 2013). Meanwhile, the decline of transcytosis in this SI site on DPW2 could contribute to a prevention of an antigen overload (Wijtten *et al.*, 2011). Both distal paracellular and transcellular permeation returned to the preweaning levels on DPW5 in NBW piglets, which is in agreement with the widely reported weaning-induced distal SI barrier function loss that takes place between DPW1 and DPW5 (Spreeuwenberg *et al.*, 2001; Boudry *et al.*, 2004; Moeser *et al.*, 2007a; Moeser *et al.*, 2007b; Wijtten *et al.*, 2011). However, as a response to weaning stress, these distal SI permeability alterations were not observed in IUGR piglets. From this it is clear that postweaning alterations in paracellular and transcellular permeability are largely site and type of piglet (IUGR vs. NBW) dependent.

Besides intestinal permeability, IUGR also affected the weaning induced villus atrophy process. As a result of reduced villus height and crypt hyperplasia, the low V/C ratio is a widely used indicator of SI integrity loss (Keating *et al.*, 1995). A surprising finding in this study is that NBW piglets showed a lower V/C ratio than IUGR ones on DPW5, which suggests that IUGR piglets may have better

nutrient absorptive capacity than NBW piglet at this time point. However, this difference was transient, since on DPW12, there was a tendency that IUGR showed a lower V/C ratio than age-matched NBW controls. To note, Boudry *et al.* (2011) stated that differences in villi and crypt surface areas could account for the potential differences in permeability. However, regarding IUGR piglets, whether the increased transcytosis is associated with morphological alterations is not clear. Further studies are needed to address this point. Taken together, both the intestinal permeability and mucosal architecture results suggest that piglets born with IUGR display different intestinal adaption process to weaning as compared to NBW piglets, probably due to the less prominent effect of weaning aneroxia.

4.2 IUGR did not affect intestinal redox status represented by GSSG/GSH ratio in weaned piglets

The GSH/GSSG redox couple is known to modulate intestinal cell transition through proliferation, differentiation or apoptosis and to govern the regenerative potential of mucosa (Circu & Aw, 2012). In the present study, although IUGR piglets showed higher proximal GSH concentrations on DPW0 in proximal SI, and higher distal SI GSH concentration on DPW5 and DPW28 than NBW age-matched littermates, neither alterations in morphological nor barrier function could be related with the corresponding alterations of GSH/GSSG redox couple, indicating that weaning induced intestinal dysfunction could not be regulated through adjusting the GSSG/GSH ratio.

4.3 IUGR affected the proximal SI redox-sensitive gene expression

Since major differences between IUGR and NBW piglets were found in the proximal SI, expression of redox-sensitive genes were further investigated in this site. In our previous study, we have demonstrated that neonatal piglets with IUGR were not able to perform the similar antioxidant defense in the proximal SI as NBW piglets at the end of neonatal period (Wang *et al.*, 2016). Accordingly, in the current study, we found significantly lower expression of *SOD1* and *CAT* on 19 day of age in IUGR piglets, compared to age-matched suckling NBW piglets. After weaning, *CAT* expression was higher

in IUGR piglets on DPW5 and DPW28 than NBW piglets, suggesting that the upregulation of *CAT* might be postponed in IUGR piglets.

In line with previous studies, the increased *TNF-α* expression in NBW piglets of this study demonstrates that weaning is associated with inflammation of the SI (Pie *et al.*, 2004). Compromised intestinal mucosal barrier by acute fasting may allow passage of luminal antigens into lamina propria where an inflammatory response can be started (McCracken *et al.*, 1999). Therefore, the unchanged HRP permeability during the first 5 days postweaning forced us to speculate that in current study, the weaning anorexia, but not diet shift, was contributing to the intestinal inflammation (McCracken *et al.*, 1999). Additionally, considering that TNF-α is a proinflammatory cytokine not only involved in increasing TJs permeability but also enhancing transcellular transport (Ménard *et al.*, 2010), the sudden increased HPR permeability on DPW12 as compared to the preweaning level in NBW piglets might be a consequence of the intestinal inflammation. Therefore, although the feed intake was resumed on DPW5 to DPW12, the *TNF-α* expression still kept at the high level on DPW12 in NBW piglets. In IUGR piglets, the lower *TNF-α* expression compared to NBW piglets on DPW2 indicate that the higher HRP fluxes could not be attributed to TNF-α mediated regulation. In this regard, the result of *TNF-α* mRNA level supports that the enhanced transcytosis on DPW2, as compared to preweaning level in IUGR piglets could be a reflection of a less mature intestine rather than a weaning induced response. In addition, TNF-α is one of the most potent physiological inducers of NF-κB, which is involved in the transcription of several key antioxidant enzymes like *GPX1* and *TXNRD1* (Chandel *et al.*, 2000). In NBW piglets, the upregulation of *GPX1* and *TXNRD1* on DPW5, as compared to the preweaning level suggests that the weaning-stress induced *TNF-α* upregulation triggered NF-κB activation. In IUGR piglets, *GPX1* and *TXNRD1* expressions were both lower on

DPW5 as compared to NBW ones, which is in agreement with the lower TNF-α expressions on the first twelve days after weaning, may indicate a less expression of NF-κB compared to the NBW piglets.

Hydrogen peroxide (H_2O_2) is another important signaling molecule for NF-κB activation. CAT catalyzes the decomposition of H_2O_2 to water and oxygen (Chelikani *et al.*, 2004). It has been stated that TNF-α can downregulate *CAT* expression, to keep sufficient H_2O_2 concentration for appropriate activation of the NF-κB, to upregulate the NF-κB dependent antioxidant gene expression (Lupertz *et al.*, 2008). Likewise, *HMOX1* could inhibit TNF-α-dependent activation of NF-κB expression (Banning & Brigelius-Flohé, 2005). Accordingly, the lower mRNA level of *CAT* and *HMOX1* on DPW5 in NBW piglets corroborate the upregulation of *GPX1* and *TXNRD1* expression as compared to IUGR ones, likely through regulating NF-κB activation. VNN1 acts as a PPARG antagonist *in vivo* and it exerts proinflammatory activity and promotes oxidative stress through PPARG inhibition (Berruyer *et al.*, 2004; Berruyer *et al.*, 2006; Dammanahalli *et al.*, 2012). The higher expression of *PPARG* on DPW5 in NBW piglets indicates that the antioxidant response can be also mediated by PPARG. Importantly, most of the above-mentioned genes upregulation was observed on DPW12 in IUGR piglets, corroborating the increased *TNF-α* mRNA level from DPW5 to DPW12, indicating that the TNF-α dependent regulation of redox-sensitive genes was postponed in IUGR. Taken together, the findings on transcriptional level provide another line of evidence that IUGR may have delayed intestinal protective responses to stress.

5 CONCLUSION

In NBW piglets, we found that weaning induced alterations in intestinal architecture are accompanied by upregulation of *TNF-α* expression, which may subsequently have triggered increased intestinal transcytosis. Meanwhile, the stable GSSG/GSH ratio suggest that the intestinal redox status is not related to these alterations. The change in transcriptional pattern of antioxidant genes in the proximal

SI of NBW piglets suggests an activation of NF-κB protective pathway. Meanwhile, the weaning induced proximal SI architecture changes and barrier function loss, as well as transcriptional antioxidant responses were delayed in IUGR piglets. Our results add to the view that IUGR piglets may need special nutritional intervention due to the particular intestinal response to stress.

Chapter IV

Effect of intrauterine growth restriction on glutathione redox cycle of weaning piglets when chronically challenged by oxidized fat

ABSTRACT

Mounting evidence shows that intrauterine growth restricted (IUGR) offspring exhibit attenuated antioxidant responses. However, the nature of the oxidative challenge is not always well defined. The consumption of oxidized fat has been proven to induce oxidative stress and redox imbalances. In this study, the hypothesis was that the exposure to oxidized fat will differently affect the antioxidant glutathione redox cycle in case of IUGR. Weaned piglets born with normal birth weight (NBW) or IUGR were randomized to diets containing non-oxidized or oxidized linseed oil. After 5 or 28 days, the glutathione/glutathione disulfide (GSH/GSSG) redox couple, as well as activity and transcription of key enzymes involved in the GSH redox cycle were evaluated in liver, small intestine and blood. Results showed that during the first 5 days after weaning, weaning induced anorexia is prominent in NBW piglets as that in IUGR piglets. From day 5 on, feed intake of IUGR piglets were significantly lower than that of NBW piglets ($P < 0.05$). Nonetheless, the more oxidized state of GSH/GSSG redox couple and a lower level of GSH in the proximal SI in IUGR piglets, as compared to NBW ones, suggests an attenuated GSH dependent antioxidant defense. Moreover, it seems that IUGR resulted in a greater entry of lipid peroxidation end-products in the circulation than the NBW piglets, and desregulation of GSH dependent enzymes both in the proximal SI and liver. Finally, the regulation pattern of redox-sensitive enzymes involved in GSH redox cycle on transcriptional level were different between IUGR and NBW piglets. All these results suggest that offspring born with IUGR may need specific nutritional care to compensate for less effective endogenous antioxidant response.

1 INTRODUCTION

Intrauterine growth restricted (IUGR) offspring is defined as fetus or newborn that does not reach its growth potential at corresponding gestational age (Wu *et al.*, 2006). Besides the well documented association between IUGR and high rates of perinatal morbidity and mortality, IUGR has also been reported to catalyze susceptibility to metabolic syndromes associated with disturbed redox status (Wang *et al.*, 2008a; Rueda-Clausen *et al.*, 2011). Our previous study on neonatal pigs have shown that when birth oxidative stress occurs, IUGR neonates seemed to be incapable to trigger proper antioxidant response in the small intestinal mucosa, as indicated by lower mRNA level of heme oxygenase 1 (*HMOX1*), catalase (*CAT*) and thioredoxin reductase 1(*TXNRD1*), compared to normal birth weight (NBW) counterparts (Wang *et al.*, 2016). Moreover, Wang *et al.* (2010; 2014) found a reduced expression of protein disulfide isomerase-associated 3, and increased expressions of peroxiredoxin-1, -5, and glutathione S-transferase (GST), compared to the NBW piglets before and after birth, stating that the redox-sensitive signaling pathways may be attenuated in IUGR piglets (Wang *et al.*, 2010). Importantly, we observed downregulated glutathione peroxidase 1 (*GPX1*) mRNA level in proximal small intestine (SI) mucosa after birth, and lower GPX activity in plasma after weaning in IUGR piglets (Michiels *et al.*, 2013; Wang *et al.*, 2016). Additionally, Che *et al.* (2015) reported a decreased GPX activity in liver of IUGR neonatal piglets (Che *et al.*, 2015), indicating an attenuated glutathione (GSH) dependent antioxidant function in IUGR piglets. These results suggest that compromised redox homeostasis may happen in IUGR offspring when oxidative challenge is present. However, in these studies, the nature of the oxidative challenge is not always well understood or defined, thus many other factors such as intermediate metabolism or feed consumption level could have interfered. Therefore, in the current study, IUGR subjects and NBW counterparts were chronically exposed to dietary oxidized fat (OF), to evaluate the effect of IUGR on the GSH redox cycle.

The consumption of hypercaloric western diets plays an important role in some diet-related chronic diseases associated with oxidative stress (Rueda-Clausen *et al.*, 2011; Heinonen *et al.*, 2014; Riccio & Rossano, 2015). As components of heated or fried foods, OF is the main inducer of several deteriorative changes in chemical, sensory and nutritional properties of foods (Dobarganes & Marquez-Ruiz, 2003). One of the most frequently reported effects of OF is the induction of oxidative stress and redox imbalance, evidenced by elevated concentrations of lipid peroxidation products, and depletion of exogenous and endogenous antioxidants in tissues (Aw, 2005; Varady *et al.*, 2011; Awada *et al.*, 2012; Shen *et al.*, 2015). Additionally, dietary OF has been reported to activate the redox sensitive transcriptional factors Nrf2 and NF-κB in liver and intestinal mucosa in pig and mouse models (Varady *et al.*, 2011; Varady *et al.*, 2012). Since the intestinal mucosa is constantly challenged by diet-derived oxidants, as well as endogenously generated reactive oxygen species (ROS), it needs to maintains high antioxidant concentrations, such as GSH, as a defense mechanism (Aw, 1999; Wu *et al.*, 2004b; Aw, 2005; Lu, 2013). GSH, γ-glutamylcysteinylglycine, is a tripeptide present in most mammalian cells in thiol-reduced (GSH) or disulfide-oxidized (GSSG) form, particularly in organs with intense exposure to exogenous toxins, such as liver and intestine (DeLeve & Kaplowitz, 1991). GSH has key functions in protective processes, for instance, detoxification of electrophiles (catalyzed by glutathione S-transferase, GST), reductions of peroxides and radicals (by virtue of glutathione peroxidase, GPX) and maintenance of redox status (GSSG/GSH ratio) (DeLeve & Kaplowitz, 1991; Lu, 1999). There is constant turnover of GSH in the body, and normal cellular GSH homeostasis is maintained through de novo synthesis from sulfur-containing precursor amino acids, through regeneration from GSSG, and through GSH uptake from exogenous sources via Na+-dependent transport systems (Wu *et al.*, 2004b; Lu, 2009). The synthesis of GSH is a two-step reaction, and the rate-limiting step in the synthesis is the glutamyl-cysteine ligase (GCL) catalyzed reaction, which is subject to feedback regulation by GSH (Deneke & Fanburg, 1989). Numerous studies have shown

that dysregulation of GSH redox cycle happens during chronic oxidative injuries (Tsunada *et al.*, 2003; Iles & Liu, 2005; Deponte, 2013; Lu, 2013). Importantly, due to the function of GSH in GPX catalyzed reduction of lipid hydroperoxides, it has been suggested that the intestinal mucosal GSH could ultimately determine the metabolic fate of dietary peroxidized lipids (Wendel & Feuerstein, 1980; Aw, 1994).

Given the importance of GSH in intestinal antioxidant defense, we hypothesized that piglets could face oxidative stress after chronic consumption of OF, but the antioxidant defense represented by GSH redox ratio and regulation of associated enzymes would also be disturbed by IUGR. The objective of the present study was to determine whether there is a difference of intestinal GSH dependent antioxidant responses between IUGR and NBW piglets consuming OF diet. Also, since the biosynthesis of GSH occurs predominantly in the liver, with subsequent release of GSH across the sinusoidal membrane into plasma, and hepatocytes can actively export GSSG under oxidative stress, the concentration of GSH/GSSG redox couple were also investigated in liver and plasma.

2 MATERIAL & METHODS

2.1 Oxidized linseed oil and diets

Linseed oil was used as the fat source in this study. Non stabilized linseed oil was purchased from Mosselman (Ghlin, Belgium). One part of the linseed oil was directly stabilized by the addition of 100 mg/kg butyl hydroxytoluene (BHT). To the other part of linseed oil, the following oxidants were added: 143.5 mg/kg $FeSO_4.H_2O$, 58.94 mg/kg $CuSO_4.5H_2O$, 600 mg/kg H_2O_2 and 3 mL/kg of H_2O. Then the oil was heated to 55°C and under continuous agitation and exposed to air at 0.25 bar for 276 h. Peroxide value (POV) of the linseed oil was measured by redox titration method. The sample was first dissolved in mixture of chloroform and acetic acid (V:V/1:2), then by flowing nitrogen gas through the sample to dispel residual oxygen. Subsequently, potassium iodide was added, and free iodine with titrated with 0.01 mol/L sodium thiosulfate. The endpoint is determined by the maximum inflexion

point on titration curve. POV is calculated from titration volume of sodium thiosulfate. The POV was monitored daily until the target level (225 mEq. O_2/kg oil) was achieved. The oxidized oil was then cooled, 100 mg/kg BHT was added and kept at -20℃ until the preparation of the experimental diets. Two isocaloric and isolipidic diets were formulated according to nutrient and energy needs of piglets, i.e. the control diet and the OF diet (**Table IV-1**). The OF diet contained 5% of the oxidized linseed oil, while the control diet contained 4.10% of the stabilized linseed oil and 0.90% tallow to balance the fatty acids in the diet. Fatty acid analysis of the diets confirms that the two diets had similar contents of unsaturated fatty acids (**Table IV-1**).

2.2 Animals and Treatments

The experiment was carried out according to the guidelines of the Ethical Committee of Ghent University (Belgium) for the humane care and use of animals in research (EC 2013/167). Sixteen triplet littermate piglets (Topics hybrid x Piétrain) were selected on a commercial breeding farm from sixteen sows. Per triplet, one NBW piglet was allocated to the control diet (first treatment; CON) and one NBW piglet to the OF diet (second treatment; OFNBW); while the IUGR littermate received the OF diet (third treatment; OFIUGR) (n=16 per treatment). Only piglets from litters with more than 13 piglets born alive were used in this study. Piglets defined as an IUGR piglet had a birth weight between 0.75 and 0.90 kg and belonged to the lower quartile of litter birth weights, while NBW piglets were identified as piglet with a birth weight within the interval of the mean litter birth weight ± 0.5 times the standard deviation (SD) within that litter. Average birth weight for IUGR and NBW piglets in this study was 0.84 kg (SD 0.09) and 1.37 kg (SD 0.18), respectively. Piglets were allowed to suckle the sow during the lactation period (until 21d of age), then they were weaned and transferred to the experimental nursing facility (size of each pen is 4 m × 2 m × 0.8 m) and 4 piglets (either IUGR or NBW ones) were kept in one pen. Piglets were allocated to different experimental diets, which is referred as day 0 (D0) of the experiment, until sampling.

Table IV–1 *Composition of the experimental diet*

Ingredients (%)	Control diet	OF contained diet
Barley	34.94	34.94
Wheat	19.96	19.96
Soybean meal	9.98	9.98
Toasted soybean	8.98	8.98
Corn	5.55	5.55
Sweet whey powder	3.99	3.99
Sugar beet pulp	2.00	2.00
Wheat gluten meal	2.00	2.00
Lactose	2.00	2.00
Glucose	0.50	0.50
52% Lactic Acid	1.15	1.15
Calcium hydrogen phosphate dihydrate	1.10	1.10
Potato protein	1.00	1.00
Limestone	0.70	0.70
Flavouring	0.03	0.03
Trace mineral and vitamin Premix*	0.12	0.12
L-lysine HCl	0.54	0.54
DL-methionine	0.18	0.18
L-threonine	0.18	0.18
L-valine	0.05	0.05
L-tryptophan	0.05	0.05
Linseed oil	4.10	-
Tallow	0.90	-
Oxidized linseed oil	-	5.00
Caculated Nutrient Values		
NEv(1997) kcal/kg†	2567	
Crude protein (g/kg)	179	
Crude fiber (g/kg)	83.7	
Digestible lysine (g/kg)	11	
Digestible methionine +cystine (g/kg)	6.6	
Digestible threonine (g/kg)	6.7	
Digestible tryptophan (g/kg)	2.2	
Major unsaturated fatty acid (% of total fatty acids)	**22.50**	**22.60**
18:1	20.09	21.15
18:2 (n-6)	12.52	15.17
18:3 (n-3)	45.49	46.33

* The mineral and vitamin premix provided the followed ingredients to per kg diet: Vitamin A, 10000 IU; Vitamin D_3, 2000 IU; Vitamin E, 54 IU; Vitamin K_3, 1 mg; Vitamin B_1, 1.2 mg; Vitamin B_2, 3.7 mg; Vitamin B_3, 12 mg; Vitamin B6, 2 mg; Vitamin B_{12}, 0.03 mg; Niacine, 20 mg; Folic acid 0.8 mg; Fe^{2+}, 100 mg; Zn^{2+}, 100 mg; Cu^{2+}, 15 mg; Mn^{2+}, 80 mg; Se^{6+}, 0.3 mg; I^-, 1 mg; BHT, 75 mg; Ethoxyquin, 37.7mg/kg.

† Net energy for pigs CVB(1997), Centraal Veevoederbureau, Lelystad, The Netherlands.

From D0 to D5, piglets were fed ad libitum and feed intake was recorded on pen level in this adaptation period after weaning. From D6 on, piglets were fed 5 meals per day individually by a separated feeder and had free access to water. Feed intake was recorded individually. No antibiotics in feed or water were applied during the whole experimental period.

2.3 Plasma and tissue collection

All animals were sampled at a fixed time point (4 h) post-prandial. On day 5 and day 28 of the experiment, 8 piglets from each treatment were weighed and slaughtered after electronarcosis and exsanguination. Blood was collected in both EDTA treated tubes and heparinized tubes containing 1 mmol/L bathophenanthroline disulfonate sodium (BPDS). The EDTA plasma was kept in -20℃ pending malondialdehyde (MDA) analysis.

After opening the abdominal cavity, the liver was first collected. The gastrointestinal tract was removed and the small intestine (SI), defined as the part of the gastro-intestinal tract between the pylorus and the ileo-cecal valve, was obtained and its length was measured. A 20 cm segment of the proximal SI or distal SI (5% or 75% of total SI length) was emptied and carefully flushed with saline. This 20 cm segment was placed on an ice-cold surface and the mucosa was retrieved by gently scraping the mucosal surface with a glass slide. Then, the collected mucosa was transferred to a glass beaker and stirred with a spoon to obtain a homogeneous representative sample. Aliquots of mucosa were either used instantaneously for acid and phosphate buffered aqueous extracts or transferred to a 2-mL screw-capped cryovials, snap-frozen in liquid nitrogen and stored at -80 ℃ pending gene expression analysis.

2.4 Plasma and tissue homogenate extracts

500 µL of heparinized blood was transferred to 2 mL tube and centrifuged at 3000 G for 15 min. Afterwards, the supernatant was removed, 600 µL of ultrapure water (Milli-Q ultrapure water system,

Merck Chemicals N.V./S.A., Overijse, Belgium) and 100 µL of 70% metaphosphoric acid were added to the pellet, followed an intense vortex and centrifugation at 3000 G for 10 min. This acid extract was then aliquoted to different tubes containing 50 µL of internal standard γ-glu-glu (Sigma Aldrich BVBA, Diegem, Belgium), stored at -80 ℃ pending GSH/GSSG quantification.

The acid extract of the liver and mucosa was prepared as previously described (Chapter II). Samples were then snap frozen in liquid nitrogen and stored at -80 ℃ until the analysis of GSH and GSSG. In addition, a phosphate buffered aqueous extract of tissue homogenates was prepared as previously described (Chapter II), and samples were snap frozen and stored at -20 ℃ until GPX, glutamate cysteine ligase (GCL), glutathione-disulfide reductase (GSR) and MDA analysis.

2.5 Determination of GSH, GSSG and enzyme activities in GSH redox cycle

The biuret method was applied to determine the total protein content in tissues. Liver, intestinal mucosal and plasma GSH and GSSG were measured using a modified high performance liquid chromatography (HPLC) method as previously described (Chapter II).

Activity of hepatic and proximal SI mucosal GCL on day 28 was determined according to the method of White *et al.* (2003) , based on the reaction of naphthalene-2,3-dicarboxialdehyde(NDA) with the γ-glutamylcysteine(γ-GC) produced in a medium containing 40 mM ATP, 20 mM glutamic acid, 2 mM cysteine and 25 μL of sample (0.075-0.15 mg protein). Fluorescence intensity of product formed was measured at 472 nm excitation and 528 nm emission using a fluorescence plate reader (Thermo Scientific, Marietta, OH, USA). One unit of GCL is defined as nmol of NDA-γ-GC formed per minute at 25℃ (pH =7.5).

Glutathione-disulfide reductase (GSR) activity was determined using the method of Carlberg and Mannervik, based on the reduction of GSSG by NADPH in the presence of GSR (Carlberg &

Mannervik, 1985). The reaction was measured by the decrease in the absorbance at 340 nm using an extinction coefficient (ε^{mM}) of 6.22 for NADPH. One unit of GSR will cause the oxidation of 1.0 μmole of NADPH at 25℃ (pH = 7.5). GPX activity was determined by colorimetric assay described before (Michiels *et al.*, 2013) (Chapter II).

2.6 RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Since major differences in GSH redox cycle parameters were found in liver and proximal SI mucosa, gene expression was performed on liver and proximal SI mucosa. Hepatic and mucosal total RNA was isolated from snap-frozen liver or intestinal mucosa at 5% of SI length, using Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, CA, USA), including an on-column DNase treatment to remove genomic DNA (gDNA). The concentration (ranging between 300-1500 ng/µL) and purity (OD 260/280 ranging between 1.9-2.2) of RNA was measured with NanoDrop ND-1000 (Nanodrop Technologies, Thermo Scientific, Wilmington, DE, USA). Integrity of RNA was verified by loading RNA onto a 0.8% agarose gel and evaluating the 28S and 18S ribosomal RNA bands, while verification of the absence of any gDNA contamination by means of a minus RT control PCR using YWHAZ primers as previously described. Synthesis of cDNA and determination of mRNA abundance by real time quantitative PCR (qPCR) was performed as previously described. Primers used to amplify target genes in this study were designed as previously described and listed in **Table IV-2** and Chapter II. A five-fold dilution series (5 points) of cDNA was included in each run to determine PCR efficiency by constructing a relative standard curve. In this study, PCR efficiencies were consistently between 98%-104% and were used to convert the quantitation cycle values into raw data. The relative expression was expressed as a ratio of the target gene to the stable expressed reference genes, then samples having the highest expression of the target gene were used as calibrator for the normalization of raw data.

2.7 Reference gene selection

A selection of reference genes was done using 8 commonly used reference genes (*ACTB, HMBS, HPRT1, TBP, TOP2B, YWHAZ, RPL4, and PPIA*), as previously described. Primers for these reference genes were listed in detail in previous study (Erkens *et al.*, 2006). The raw data were analyzed using the geNorm algorithm (Vandesompele *et al.*, 2002). The stepwise exclusion of the reference gene with the least stable expression showed that *TBP, RPL4* and *HPRT1* were the three most stably expressed reference genes in the analyzed samples. Thus, these three genes were used as the reference genes in this study to normalize the raw data from qPCR.

Table IV–2 *Primer sequences used for RT-qPCR*[*]

Gene symbol	Accession number	Nucleotide sequence of primers (5' – 3')	Product length (bp)
GSS	NM_001244625	F: GCTATGCCCCGTTCACACTC R: AGTTGTCCCTTTTGATGGTGCT	159
GSR	AY368271	F: CTACGTGAGCCGACTGAACA R: TCAGGATGTGAGGAGCTGTG	146
GCLM	XM_001926378	F: GGACAAAACCCAGTTGGAGCA R: CAGTTAAATCGGGCGGCATC	104
GCLC	XM_003482164	F: CCAAGTCCCTCTTCTTTCCAG R: TCGCTGCTTCATCATCCTC	177
GPX1	NM_214201	F: TGCTCATTGAGAACGTAGCGT R: CAGGATCTCCCCATTCTTGGC	161
GSTA4	NM_001243379	F: AGACAAGCACCATCTCTTCGGCAA R:GCTGATCGTCTGGTTTGAGGAAAGG	125

[*]All these primer sequences were designed based on the sequence corresponding to the accession number and can only amplify the isoform listed above. GSS, GSH synthetase, GSR, GSH reductase, GCLM, GCL modify subunit; GCLC, GCL catalytic subunit.

2.8 Statistical analysis

Normality of data and homogeneity of variance were tested using the Brown-Forsyth test in SAS Enterprise Guide 7 (SAS Institute, Cary, NC, USA). Treatment effects were analyzed per sampling day with one-way analysis of variance and orthogonal contrasts (Tukey's honestly significant difference)

with the animal as experimental unit (n = 8). Data are expressed as means with pooled standard error of the mean (S.E.M), and $P < 0.05$ was considered significant.

3 RESULTS

3.1 Body weight, growth and feed intake

Table IV–2 Growth performance of IUGR and NBW piglets fed control or oxidized fat diet during the first 28 days postweaning. At the beginning of the experiment, the IUGR piglets showed a 27% lower weaning weight than the NBW ones ($P < 0.01$). During the first 5 days postweaning, except the CON group, both OFIUGR and OFNBW piglets showed a negative growth, the average daily gain (ADG) did not differ between treatments ($P > 0.05$). Feed intake was lower ($P < 0.001$) for OFIUGR than in NBW piglets, but when expressed per kg mean body weight, the feed intake was similar between IUGR and NBW piglets feeding OF diet.

Table IV–2 Growth performance of IUGR and NBW piglets fed control or oxidized fat diet during the first 28 days postweaning

	TREATMENT				
	CO	OFNBW	OFIUGR	S.E.M.	*P* values
Body weight					
Initial body weight at day 0 (kg)*	6.05[a]	6.14[a]	4.45[b]	0.14	< 0.001
Body weight at day 5 (kg)	6.06[a]	5.82[a]	4.13[b]	0.15	< 0.001
Body weight at day 28 (kg)†	9.29[a]	8.19[b]	5.84[c]	0.36	< 0.001
Growth and feed intake					
Average daily gain day 0-5 (g/d)	1	-64	-64	0.01	0.081
Average daily gain day 5-26 (g/d)†	130[a]	89[b]	71[b]	0.01	0.004
Daily feed intake day 0-5 (g/d)‡	85.3[a]	62.3[b]	79.3[ab]	4.49	0.093
Daily feed intake day 5-26 (g/d)§	310[a]	228[b]	137[c]	16.32	< 0.001
Relative daily feed intake day 5-26 (g/d/ kg MBW)	40.5[a]	32.20[b]	27.09[b]	1.59	< 0.001
Feed conversion ratio day 5-26†	2.35	3.18	2.21	0.20	0.096

MBW, mean body weight during the experimental period.

* Data based on 48 piglets; n=16 per treatment.
† Data based on the remaining 24 piglets. n=8 per treatment.
‡ Data based on all 48 piglets, feed intake was recorded at pen level.
§ Data based on the remaining 24 piglets. n=8 per treatment. Feed intake was recorded individually.

3.2 GSH redox cycle and MDA content in plasma, liver and intestinal mucosa

As shown in Table IV-3 and IV-4. On day 5, few of the redox biomarkers were affected by treatment. A decreased GSH concentration in erythrocytes and lower MDA concentration in liver was observed in OFNBW piglets as compared to the CON group ($P < 0.05$). In addition, OFIUGR piglets showed a higher GSH content in erythrocytes, compared to OFNBW piglets ($P < 0.05$).

Table IV–3 *Effect of treatment on redox balance and associated markers in plasma and different tissues of IUGR and NBW piglets on Day 5* [*]

	CON	OFNBW	OFIUGR	S.E.M.	*P* values
Plasma					
GSH (nmol/mL)	320[a]	252[b]	294[a]	9.13	**<0.001**
Ratio GSSG/GSH	0.12	0.15	0.15	0.009	0.306
MDA (nmol/mL)	9.02	10.89	9.02	0.51	0.309
Liver					
GSH (µmol/g protein)	9.42	9.32	8.3	0.55	0.666
GSSG/GSH Ratio	0.06	0.07	0.06	0.004	0.523
MDA (nmol/g protein)	1484[a]	1038[b]	1163[ab]	75.1	0.029
Proximal SI mucosa					
GSH (µmol/g protein)	6.35	7.10	7.11	0.35	0.623
GSSG/GSH Ratio	0.06	0.05	0.06	0.004	0.833
MDA (nmol/g protein)	201	210	207	12.0	0.534
Distal SI Mucosa					
GSH (µmol/g protein)	4.62	5.31	6.23	0.41	0.276
GSSG/GSH Ratio	0.04	0.04	0.04	0.002	0.182
MDA (nmol/g protein)	272	286	290	19.7	0.926

[*] *P* values smaller than 0.05 are in bold.

[a,b,c] Mean values in a row with different letters are significantly different.

On day 28, compared to the OFNBW littermates, OFIUGR piglets showed lower GSH concentration in erythrocytes and proximal SI mucosa, but higher GSH content in liver (P <0.05). Furthermore, GSSG/GSH ratio in the proximal SI mucosa from OFIUGR piglets were higher than the OFNBW ones (P < 0.05). As for MDA, in the proximal SI mucosa, the OFIUGR ones showed a lower MDA content (P < 0.05). However, in plasma, the MDA content was higher in OFIUGR piglets, compared to OFNBW ones (P < 0.05). In the distal SI mucosa, neither GSH, GSSG/GSH ratio nor MDA differs between the three groups on both sampling days (P > 0.05).

Table IV–4 *Effect of treatment on redox balance and associated markers in plasma and different tissues of IUGR and NBW piglets on Day 28* [*]

	CON	OFNBW	OFIUGR	S.E.M.	*P* values
Plasma					
GSH (nmol/mL)	590[b]	747[a]	575[b]	28.2	**0.002**
Ratio GSSG/GSH	0.053	0.049	0.048	0.003	0.202
MDA (nmol/mL)	9.76[b]	10.38[b]	17.91[a]	1.29	**0.004**
Liver					
GSH (µmol/g protein)	15.36[ab]	14.37[b]	17.15[a]	0.55	**0.039**
GSSG/GSH Ratio	0.034	0.033	0.039	0.002	0.439
MDA (nmol/g protein)	1188[a]	993[b]	1113[ab]	34.9	**0.042**
Proximal SI mucosa					
GSH (µmol/g protein)	7.85[b]	9.56[a]	8.14[b]	0.43	**0.036**
Ratio GSSG/GSH	0.043[ab]	0.032[b]	0.054[a]	0.004	**0.020**
MDA (nmol/g protein)	223[b]	322[a]	241[b]	16.34	**0.021**
Distal SI Mucosa					
GSH (µmol/g protein)	4.75	5.27	4.71	0.22	0.611
GSSG/GSH Ratio	0.047	0.055	0.046	0.003	0.463
MDA (nmol/g protein)	359	354	327	26.6	0.671

[*] *P* values smaller than 0.05 are in bold.

[a,b,c] Mean values in a row with different letters are significantly different.

3.3 Enzyme activities in GSH redox cycle

The OF diet induced enhanced hepatic GSR activity, both in OFIUGR and OFNBW piglets, compared to the NBW piglets fed with control diet ($P < 0.05$; **Fig. IV-1**). Also, OFNBW piglets showed higher proximal SI mucosal and hepatic GPX activity, compared to the CON ones ($P < 0.05$). Moreover, in liver, IUGR piglets showed the highest GSR activity among the three groups, while lower GCL and GPX activity was found both in liver and proximal SI mucosa of OFIUGR ones, compared to the OFNBW ones (all $P < 0.05$).

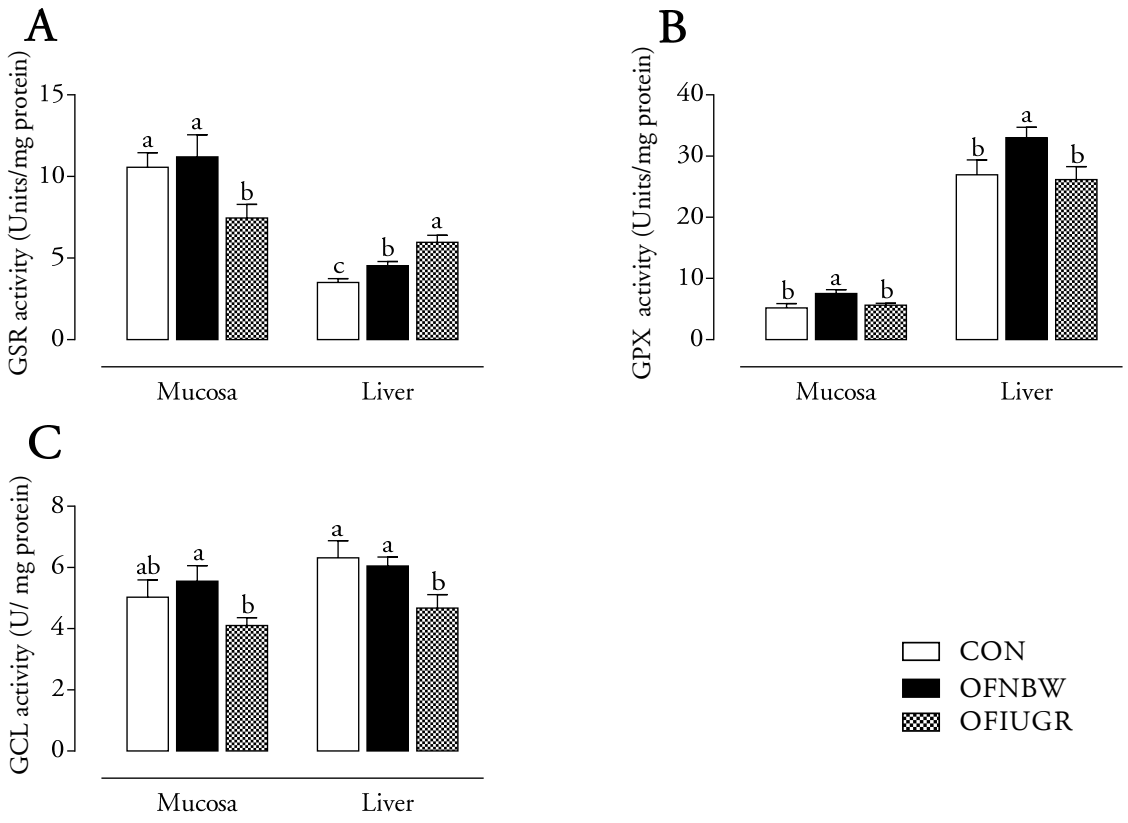

Figure IV–1 *Enzyme activities in GSH redox cycle in the proximal SI mucosa and liver on d28*

3.4 Relative mRNA level of redox-sensitive enzymes involved in GSH redox cycle

Results of transcription of redox-sensitive enzymes involved in GSH redox cycle are listed in **Fig. IV-2**. On transcriptional level, compared to NBW piglets from CON group, consumption of OF diet downregulated *GSR*, *GPX* and upregulated GCL catalytic subunit (*GCLC*) expression in the proximal SI mucosa of NBW piglets ($P < 0.05$). Meanwhile, compared to the OFNBW piglets, IUGR resulted in upregulation of *GSR,* GCL modifier subunit (*GCLM*) and GSH-S-transferase A4 (*GSTA4*) expressions, but downregulation of *GCLC* expression in liver ($P < 0.05$). Also, IUGR piglets showed lower *GCLC* and higher *GCLM* mRNA levels in the proximal SI mucosa, as compared to the OFNBW controls ($P < 0.05$). No difference of GSH synthase (*GSS*) expression were found among the three groups ($P > 0.05$).

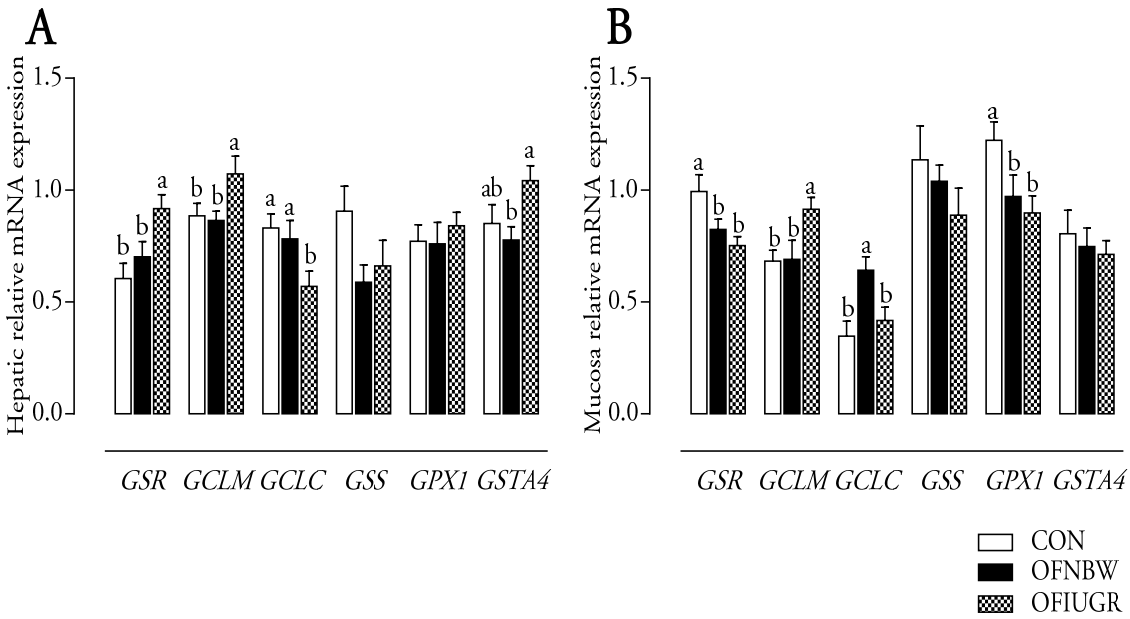

Figure IV–2 *Redox sensitive gene transcription in GSH redox cycle in the proximal SI mucosa and liver on d28*

4 DISCUSSION

This is the first study showing that IUGR could affect the GSH dependent response to OF diet-induced oxidative challenge. Previous studies have suggested that lipid peroxide-induced GSSG/GSH redox imbalance occurred within 2 weeks after peroxide challenges and thereafter tissue redox homeostasis was restored despite continued peroxide exposure and elevated tissue peroxide levels (Tsunada et al., 2003). Similarly, in the current study, short-term oxidized fat intake (5 days) barely affected the redox status in liver and SI mucosa in both IUGR and NBW piglets. Only lower MDA concentration in liver and GSH concentration in erythrocytes were observed in OFNBW piglets as compared to the CON NBW ones. However, since weaning stress induced low feed intake in the two groups of NBW piglets during the first 5 days of the experimental period, the lower MDA and GSH concentration could also result from the weaning anorexia. Although the feed intake during day 5 to day 28 was significantly lower in OFNBW piglets than the CON piglets, 4-week consumption of OF diet induced elevated proximal mucosal MDA and GSH contents, as well as erythrocyte GSH content in the OFNBW piglets, as compared to the CON ones, indicating an occurrence of oxidative injury. Moreover, the intake of OF diet upregulated GPX activity in mucosa of OFNBW piglets, compared to the piglets in CON group, suggesting the antioxidant system was activated in response to the oxidative challenge. Thus, we assume that the long-term intake of OF diet can induce oxidative challenge. Therefore, whether the GSH redox cycle under oxidative stress is different between IUGR piglets and NBW piglets can be investigated with this approach. In this respect, results of the current study can be summarized as follows: 1) IUGR piglets exhibited an attenuated GSH dependent antioxidant response in proximal SI, giving rise to increased levels of lipid peroxidation products in the circulatory system and lower erythrocyte GSH; 2) IUGR could affect the transcription of redox enzymes in the GSH redox cycle, however, in different patterns in the liver and proximal SI mucosa.

4.1 IUGR affected GSH storage and lipid peroxidation in the proximal SI

The intestine is the primary site of nutrient absorption and first defense barrier against oxidative damage induced by luminal oxidants (Aw, 2005; Circu & Aw, 2012). Accumulation of oxidants like peroxidized lipids in the gut lumen can impair the mucosal metabolic pathways, result in dysfunction (Aw, 2005). Decomposition of the unstable peroxides derived from polyunsaturated fatty acid results in the formation of MDA, a widely used marker for screening and monitoring lipid peroxidation (Yagi, 1998; Jacob *et al.*, 2003). Thus, irrespective the lower feed intake of OFNBW piglets than the CON piglets, the increased MDA concentration on day 28 in the proximal SI mucosa of OFNBW piglets as compared to the CON piglets could indicate an oxidized fat induced oxidative stress in the proximal SI. Contrary to that, compared to the OFNBW ones, OFIUGR piglets showed lower MDA content in the proximal SI mucosa, which might be partly because of the lower feed intake ($P < 0.05$). Nevertheless, compared to the OFNBW piglets, the OFIUGR piglets showed a lower GSH concentration and higher GSSG/GSH ratio in the proximal SI mucosa, suggesting a disturbed GSH redox status. Numerous researches have implicated GSH as a key determinant in elimination of peroxides by the intestine (Wendel & Feuerstein, 1980; Yagi, 1998; Jacob et al., 2003; Aw, 2005). Aw *et al.* (1979) suggested that at a given peroxide concentration, the amount of peroxide recovered from the intestinal lumen and lymph is governed by mucosal GSH status. Specifically, the absorbed lipid hydroperoxides are efficiently metabolized and the products are removed by GSH under normal healthy conditions (Aw, 2005). In the current study, the plasma MDA concentration was significantly higher, in opposite to proximal SI mucosal MDA in OFIUGR piglets, compared to the OFNBW ones. Therefore, we assume that the lower GSH level and GPX activity in the proximal SI mucosa could contribute to this higher present of lipid peroxides in the circulation. Disturbed GSH redox status can lead to impaired mucosal peroxide removal ability, resulting in an attenuated enterocyte metabolism of luminal lipid peroxides. Consequently, absorbed lipid peroxidation products from the intestinal

lumen could have greater entry to the circulation, and increase the MDA recovery in plasma. Compared to the CON piglets, the similar MDA concentration in plasma of OFNBW piglets, but higher MDA recovery in plasma of IUGR piglets, could support the speculation of greater absorption of lipid peroxidation products in IUGR piglets. Similarly, Kanazawa *et al.* (1998) reported that when rats are given lipid peroxide at the level beyond the capacity of detoxifation in intestinal cells, it results in intestinal mucosal injury and higher recovery of lipid preoxidation products in liver (Kanazawa & Ashida, 1998). Furthermore, previous research has demonstrated that the transport rate of lipids is lower in the distal intestine than the proximal site (Yagi, 1998). This could be an indication for a different metabolism in different sites of the intestine.

4.2 IUGR affected GSH dependent antioxidant response in the proximal SI

Lipid peroxidation can induce the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in numerous cells (Wendel & Feuerstein, 1980; Tsunada *et al.*, 2003). The antioxidant function of GSH is accomplished largely by GPX-catalyzed reactions, which reduce H_2O_2 and lipid peroxides as GSH is oxidized to GSSG (Wu *et al.*, 2004b). In the present study, decreased GPX activity was found in liver and mucosa of OFIUGR piglets, compared to the OFNBW piglets, indicating an impaired antioxidant capacity. Circu and Aw (2012) reported that high cell GSH is required to support GPX function during enhanced hydroperoxide reduction , so the reduced GPX activity in the proximal SI mucosa may result from the lower concentration of GSH. GPX and GSR function as an enzyme pair in the reduction of peroxides with concomitant oxidation of GSH and the regeneration of GSH from GSSG, respectively (Aw, 2005). Normal tissue GSH homeostasis is maintained by de novo synthesis from its amino acid precursors, from regeneration of GSSG, catalyzed by GSR and from GSH uptake of exogenous sources (Tsunada *et al.*, 2003). Considering that enterocyte GSH synthesis is lower as compared to hepatocytes (Shan *et al.*, 1990), GSSG reduction could be an important mechanism for intestinal GSH maintenance under high rates of lipid peroxide reduction (Aw, 1994, 2005). The lower

GSR activity in the proximal SI of IUGR piglets, suggesting a reduced GSH regeneration ability, might be an extra evidence for the attenuated antioxidant response to oxidative challenge. Additionally, GCL is the enzyme catalyzing the first and the rate-limiting step of de novo GSH biosynthesis (Iles & Liu, 2005; Lu, 2009, 2013). The activity of GCL is lower in the proximal SI of IUGR piglets than the OFNBW ones. Therefore, these result underscore that IUGR could inhibit the GSH redox cycle in the proximal SI mucosa and lead to impaired antioxidant capacity in the proximal SI. In addition, given that redox enzymes like GPX and GSR activities are minimally modified under peroxide challenge in rats (Carlberg & Mannervik, 1985; Aw, 2005; Circu & Aw, 2012), the altered activities of these two enzymes in IUGR piglets implicate that the proximal SI of IUGR subjects might be more vulnerable to the oxidative challenge. Similarly, Kaplowitz (1981) stated that under oxidative stress, liver exports GSSG into bile in a concentration-dependent manner, whereas under basal conditions, mainly GSH is exported into bile and blood, to avoid accumulation of GSSG . Considering the finding that IUGR piglets displayed a higher proximal intestinal GSSG/GSH ratio in this study, we can speculate that after a chronic oxidative challenge, the proximal SI of IUGR piglets is rendered into a more oxidative state, either from the local peroxide challenge or from the liver exported GSSG.

4.3 IUGR affected GSH dependent antioxidant response in liver

The liver plays a central role in inter-organ GSH homeostasis, it exerts as much as 50% - 60% of its total GSH into bile (Wendel & Feuerstein, 1980; Kaplowitz, 1981; Shan *et al.*, 1990; Kanazawa & Ashida, 1998). Interestingly, although GSH redox imbalance was found in the proximal SI mucosa of IUGR piglets fed with OF diet, it was not the case for liver. Similar with the proximal SI, hepatic GPX and GCL activity was found lower in IUGR piglets, as compared to the OFNBW ones, indicating both a lower GSH synthesis ability and GSH dependent antioxidant function. However, enhanced hepatic GSR activity was found in liver of IUGR piglets, suggesting an increased GSH regeneration capacity from GSSG in liver. Therefore, despite the lower GSH storage in liver, hepatic GSSG/GSH

ratio showed no difference in IUGR subjects, compared to the NBW ones. In addition, since biliary GSH is a major contributor to the intestinal luminal GSH pool (Tsunada *et al.*, 2003), although the hepatic GSH redox balance was not affected by IUGR, the higher hepatic storage of GSH may result in a decreased exogenous GSH transport from liver to intestine, exacerbating the GSH deficiency in proximal SI.

Next to the difference in GSH couple and redox enzymes activity difference, a different pattern of regulation on transcriptional level was also found in IUGR piglets under chronic OF exposure as compared to the OF diet-fed NBW ones. GCL is composed of a catalytic or heavy subunit (GCLC), and a modulator or light subunit (GCLM), which are encoded by different genes (Lu, 2009, 2013; Weldy *et al.*, 2013). The cellular content of one or both subunits might be upregulated in response to oxidative stress (Lu, 2009). Accordingly, in the current study, proximal SI mucosal *GCLC* was 2-fold higher in OFNBW piglets, as compared to the CON ones. This indicates that exposure to OF triggered the intestinal antioxidant defense. However, when being fed with OF diet, IUGR piglets showed lower *GCLC* mRNA level both in the proximal SI mucosa and in liver as compared to the OFNBW ones, suggesting an inadequate molecular response to the oxidative challenge. In contrast, *GCLM* expression showed an upregulation in both liver and the proximal SI in IUGR piglets as compared to the OFNBW ones, implicating that the regulation of these two subunits is differently affected by IUGR. Other lines of evidence are consistent with the possibility of different regulation pattern of the two GCL subunits. For example, 4-Hydroxynonenal is a lipid peroxidation product, which has been proven to dramatically increase the expression of the *GCLM*, but has only a small effect on *GCLC* (Yang *et al.*, 2002). However, it is the first time that an upregulation of *GCLM* and a downregulation of *GCLC* was found in IUGR piglets. Based on the previous studies on GCL regulation, it was suggested that the regulation of the two subunits of GCL is a complex process that can be modulated by multiple stimuli including different pathways (Yang *et al.*, 2002; Zhang *et al.*, 2007; Lu, 2013; Weldy *et al.*, 2013). Therefore,

the pathways involved in the regulation of this two units of GCL could be important to understand the molecular mechanism of GSH synthesis under oxidative stress. GSS is the second enzyme of GSH synthesis next to GCL. The transcriptional regulation of GSS is important in determining the overall GSH synthetic capacity, especially in non-hepatic tissues (Lu, 2009). Some treatments, like ethanol feeding *in vivo* in rats, could increase the expression of *GCLC* alone, but had no influence on *GSS* expression. Similarly, the present study showed that neither OF diet nor IUGR had effect on *GSS* expression. Furthermore, the enhanced GSR activity may result from an increased transcription of *GSR* mRNA in the liver of IUGR piglets, while in the proximal SI mucosa *GSR* gene expression showed no difference between IUGR and NBW piglets fed the OF diet. The *GSTA4* isoform exhibits remarkable chemo-selectivity and high catalytic efficiency towards hydroxynonenal (Balogh *et al.*, 2010). Interestingly, in liver of IUGR piglets, a 1.5-fold increased expression of GSTA4 was found as compared to the OF diet feeding NBW piglets, which could be a response to the higher MDA content in liver. These different pattern of regulation of redox enzymes on transcriptional level in IUGR offspring warrants further attention.

5 CONCLUSION

This study has demonstrated that long-term intake of OF could induce oxidative challenge in piglets. In addition, the comparison between OF-fed IUGR and OF-fed NBW piglets provides strong evidence that IUGR attenuates antioxidant defense responses to this challenge, compared to NBW piglets, illustrated by the changes in the proximal SI GSH redox cycle. Although the feed intake was lower in IUGR piglets, the intake of OF resulted in higher circulatory levels of peroxidation products, which might predispose IUGR subjects to pathologies related to oxidative damaging processes. Together with the previous studies from our group (Michiels et al., 2013), these results illustrate that IUGR may inhibit the intestinal mucosal antioxidant defense capacity, especially in the proximal SI. Our results suggest that offspring born with IUGR need special attention on nutritional intervention to avoid undue challenge to the poor antioxidant system.

Chapter V

General Discussion

1 INTRODUCTION

Selection of highly prolific sows over the years has resulted in an increase in litter size (Quiniou *et al.*, 2002). However, due to the limited uterine capacity of the sow, this increased litter size is accompanied by a significant within-litter variation in birth weight (Yuan *et al.*, 2015). The low birth weight is a characteristic of neonates that have experienced IUGR. Piglets born with IUGR represent a substantial portion of perinatal morbidity and mortality, even the survival ones show reduced growth performance during later life (Winters *et al.*, 1947; Sharpe, 1966; Wootton *et al.*, 1983; Pettigrew *et al.*, 1986; Aucott *et al.*, 2004; Guilloteau *et al.*, 2010; Wu *et al.*, 2010a). Although management techniques have been implemented for piglets born with IUGR, it is still a significant problem because of the incomplete knowledge concerning the mechanism of the retarded postnatal performance. The GIT plays a critical role in nutrient acquisition, and the intestinal epithelium is the first line of defense to prevent invasion of pathogens (Guilloteau *et al.*, 2010). Moreover, since the GIT is a major source of ROS (Bhattacharyya *et al.*, 2014), the intestinal epithelium is prone to oxidative damage, resulting in loss of barrier function, which plays a crucial role in a variety of gastrointestinal disorders (Vandervliet & Bast, 1992).

Mounting evidence has revealed that the postnatal growth retardation in IUGR piglets is associated with delayed intestinal development. For example, relatively longer and thinner intestinal tissue, reduced mucosal absorptive surface and shorter villi (Sankaran & Kyle, 2009; Guilloteau *et al.*, 2010; D'Inca *et al.*, 2011). It was also shown that IUGR piglets exhibit a low oxidative defense capacity both in the neonatal and postweaning period (Wang *et al.*, 2010; Michiels *et al.*, 2013). Therefore, it is interesting to know whether the IUGR condition has an effect on the intestinal barrier function and redox status during the early life of piglets and if there is a link between intestinal dysfunction and redox status in IUGR piglets. The aim of this Ph.D. research was therefore to contribute to a better understanding of the development of the intestinal barrier function, redox status and antioxidant

system in IUGR piglets during critical periods in their early life such as birth and weaning (**Chapter II and Chapter III**). To this purpose, the small intestinal mucosal architecture and barrier function were investigated by evaluating the intestinal villi/crypt histomorphology and *ex vivo* mucosal permeability at different time points during the pre- and postweaning period. The intestinal redox status was assessed by measuring intestinal GSSG/GSH ratio and MDA concentration. Moreover, expressions of redox-sensitive genes were determined by RT-qPCR. During the first two experiments, intestinal GSSG/GSH ratio was neither affected by IUGR, nor intestinal morphological and functional development. To further investigate the effect of IUGR on SI GSH metabolism under chronic oxidative challenge, we introduced oxidized fat as oxidant source to evaluate the intestinal redox status (**Chapter VI**). To note, in the current study, IUGR piglets were selected under strict criteria to make sure that they are the smallest ones from a relatively large litter by selecting in the lower quartile in a litter size with more than 14 live borns, and having a birth weight less than 1 kg. Using these criteria, the selected piglets were probably the ones that experienced growth restriction in utero, leading to retarded growth later in life. Throughout this thesis, the animal growth results confirmed that these selected IUGR piglets showed indeed lower growth than the NBW piglets. Therefore, we think these criteria are successful to be an IUGR biological/zootechnical justification. Moreover, a lower bound of birth weight more than 0.75 kg was applied, to make sure that the selected piglets can survive during the experimental period, and thus from the farmers' point of view interesting to rear.

In **Fig. V-1,** significant differences between IUGR and NBW piglets found in Chapter II and Chapter III are summarized, in **Fig. V-2** and **Fig. V-3,** the development of intestinal barrier function and antioxidant system in IUGR and NBW piglets during the early life is summarized respectively. Finally, in **Fig. V-4**, significant differences in GSH redox cycle between weaned IUGR and NBW piglets when challenged by oxidized fat containing diet were summarized.

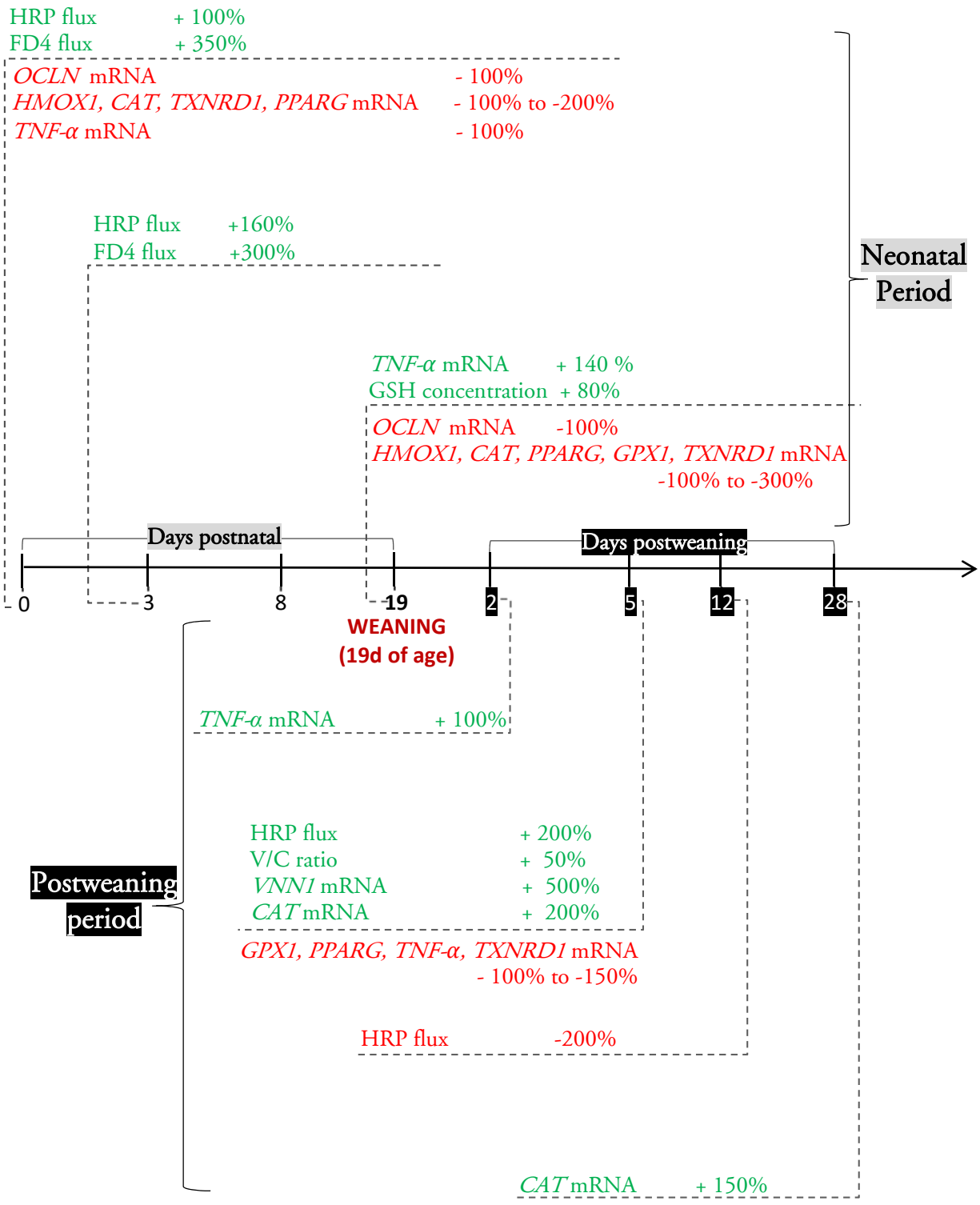

Figure V–1 *Significant differences in the proximal SI of IUGR piglets compared with age-matched NBW ones (percentile change relative to the value of NBW piglets) at different days postnatal and postweaning. Red font and green font means the parameter is downregulated or lower and upregulated or higher respectively in IUGR piglets. Detailed information can be found in Chapter II and Chapter III.*

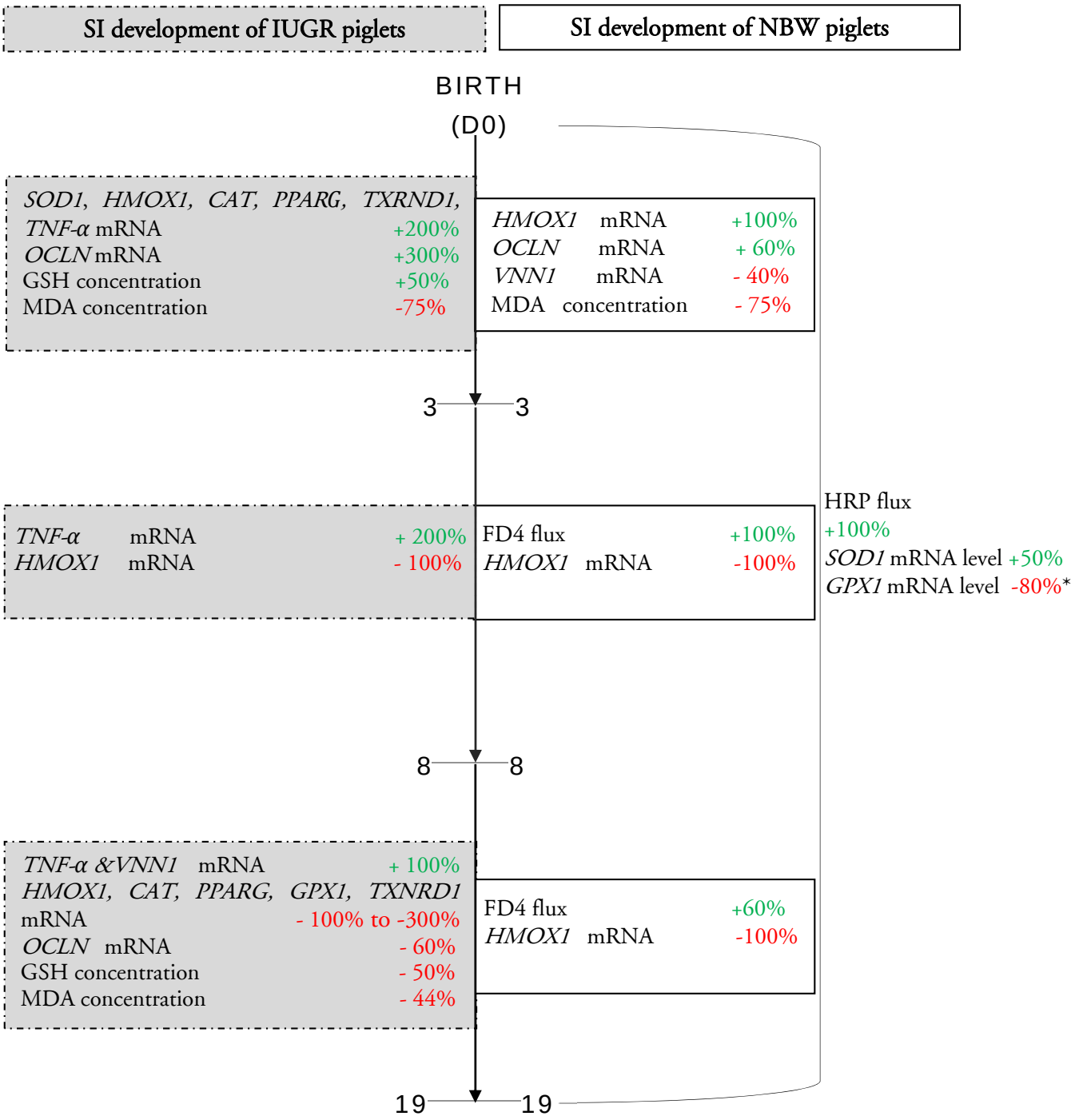

Figure V–2 *Significant differences in the proximal SI between different time points of the neonatal period in IUGR and NBW piglets (percentile change relative to the previous time point). Red font and green font means the parameter is downregulated or lower and upregulated or higher respectively compared to the previous time point. * Difference only found between DPN0 and DPN19. Detailed information can be found in Chapter II.*

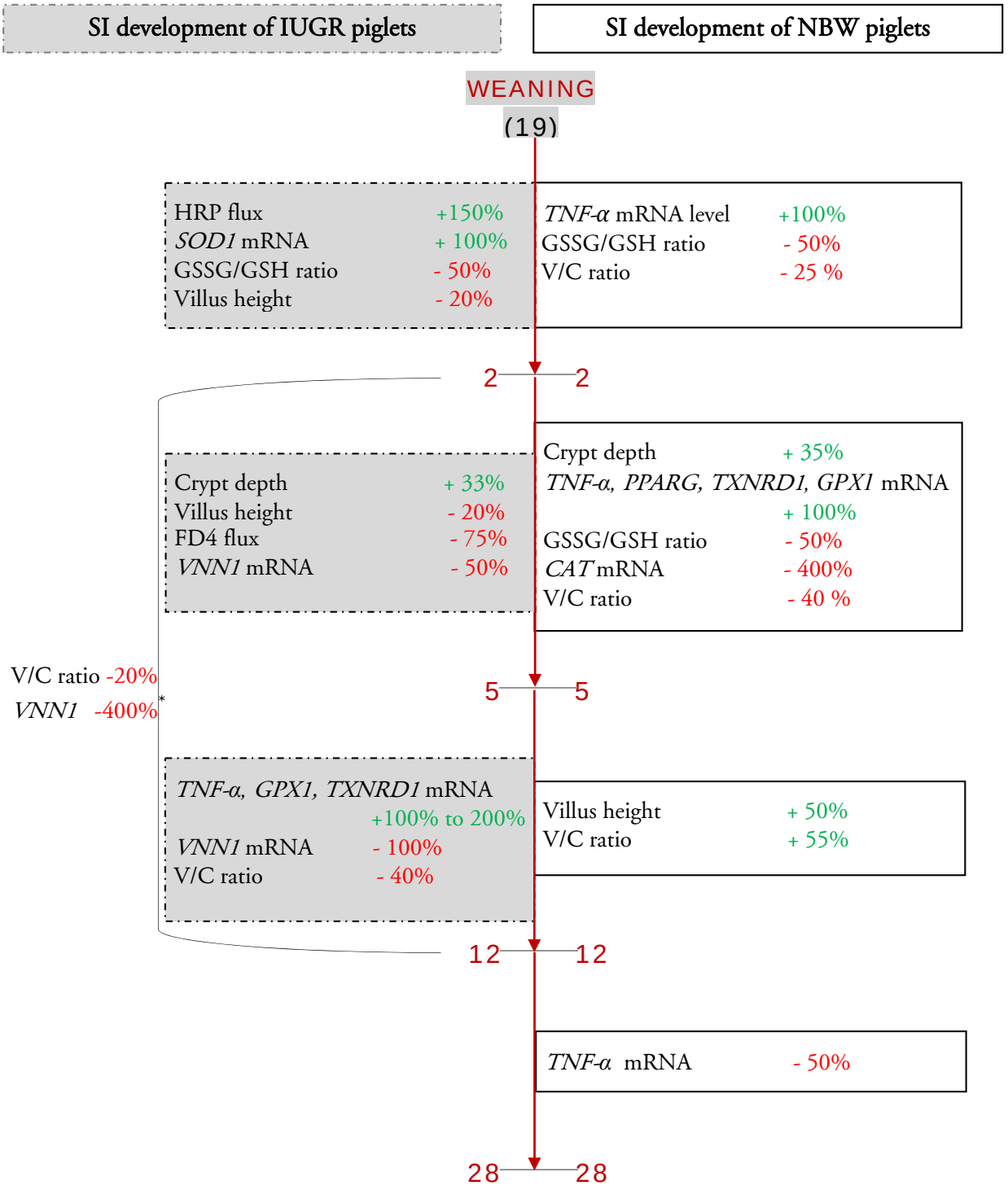

Figure V–3 *Significant differences in the proximal SI between different time points during the postweaning period in IUGR and NBW piglets (percentile change relative to the previous time point). Red font and green font means the parameter is downregulated or lower and upregulated or higher respectively compared to the previous time point. * Differences were only found between DPW2 and DPW12. Detailed information can be found in Chapter III.*

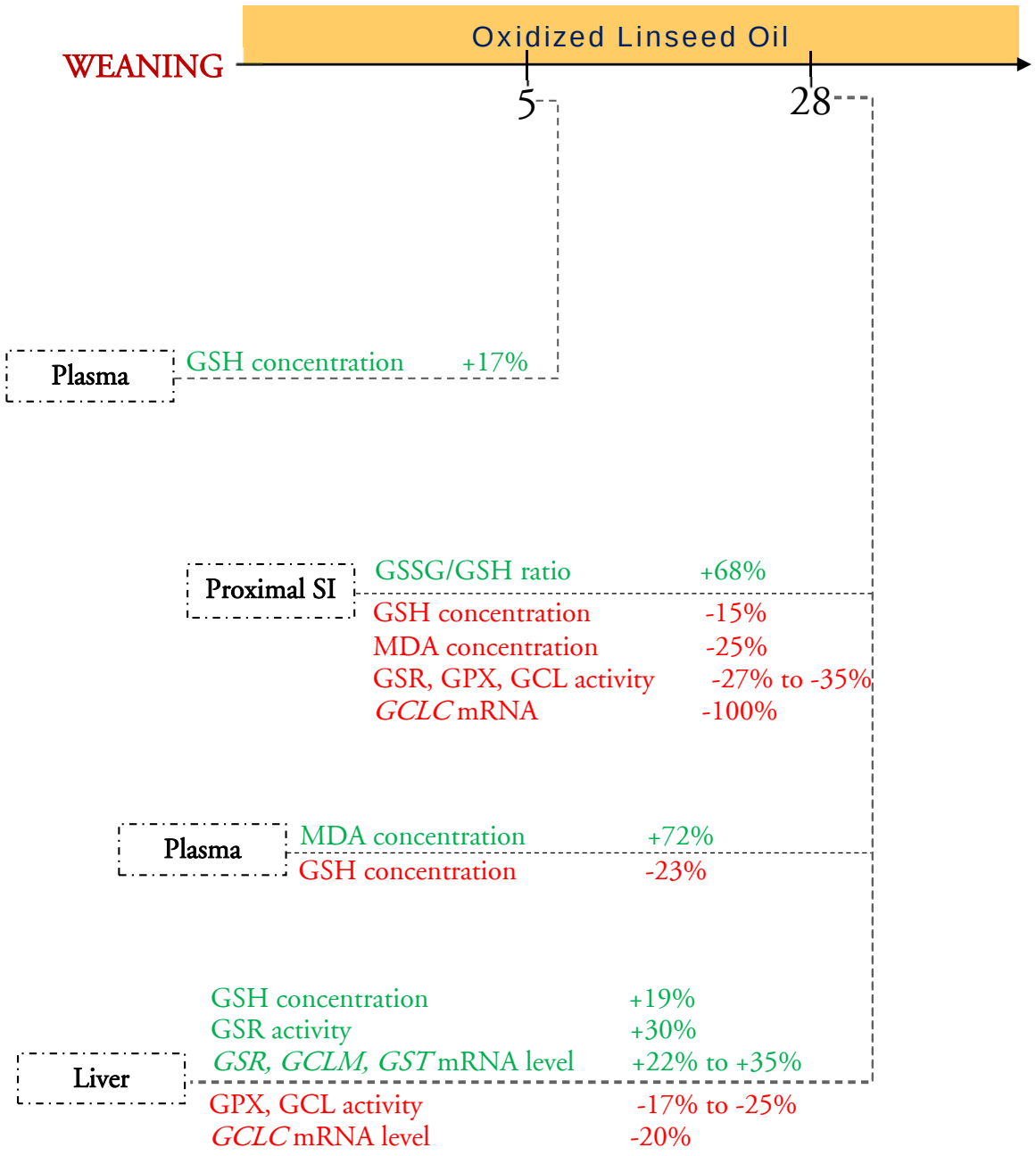

Figure V–4 *Major differences in the proximal SI, plasma and liver of IUGR piglets compared with age-matched NBW ones (percentile change relative to the value of NBW piglets) at day 5 and day 28 of experiment. Red font and green font means the parameter is downregulated or lower and upregulated or higher respectively in IUGR piglets. Detailed information can be found in Chapter IV.*

2 EFFECT OF IUGR ON INTESTINAL BARRIER FUNCTION DURING EARLY LIFE OF PIGLETS

The intestinal barrier prevents the entry of pathogens from the intestinal lumen into the blood. Compared with NBW littermates, neonatal IUGR piglets show an immature intestinal function during the early period of life (Boudry *et al.*, 2006; D'Inca *et al.*, 2011; Boudry *et al.*, 2014). In Chapter II, we demonstrated that IUGR could affect both paracellular and transcellular permeability around birth (DPN0, DPN3) in the proximal SI. In the distal SI, when compared to NBW piglets, only HRP flux was higher in IUGR piglets at birth. Using the sugar (lactulose-mannitol) absorption test (SAT), different results were found. Birth weight did not affect the intestinal barrier function during the whole neonatal period (DPN0, DPN3, DPN10 and DPN28) (Huygelen *et al.*, 2014). In contrast to the *ex vivo* intestinal barrier assessment in our study, such *in vivo* SAT assessment considers mannitol as a marker of the transcellular pathway, whereas lactulose is the marker for paracellular pathway (Ménard *et al.*, 2010). This *in vivo* SAT assesses the permeability of the entire SI, however, could not measure the barrier function at a specific site of the SI. An apparently opposite finding to the one in our study was also noticed in a study assessing *ex vivo* mucosal permeability of cephalexin (CFX) in Ussing chamber, with CFX fluxes across the proximal jejunum mucosa being similar in IUGR and NBW piglets on DPN2 (Boudry *et al.*, 2014). Similar to HRP, CFX1 is also a maker for transcellular permeability. CFX crosses the epithelium through the peptide transporter 1(PepT-1)-mediated pathway and is unlikely through a paracellular leak. Taken these results together, it seems that IUGR affects the intestinal barrier function in a site-specific manner. In addition, Boudry *et al.* (2006) reported that intestinal permeability of FD4 showed no difference between IUGR and NBW littermates on DPN7 and DPN28, which agrees with our result that the effect of IUGR on barrier function only happened transiently in the early days postnatal.

Intestinal mucosal barrier function can be partly affected by the mucosal structure (Boudry *et al.*, 2014), so we checked if the observed permeability difference on DPN0 and DPN3 between IUGR and NBW piglets was related to differences in the mucosal morphology of the proximal SI. Villus height, villus surface area, and crypt depth were similar for the two categories of piglets, suggesting that the mechanism involved in the increased intestinal mucosal permeability in the IUGR piglets is not related to the mucosal architecture of the proximal SI. Another critical factor in the maintenance of gut permeability and intestinal barrier function is the intercellular contacts between epithelial cells formed by mucosal TJs (Clark *et al.*, 2006). Occludin is a unique marker of TJs, which helps to connect TJs proteins and cytoskeletons. In accordance with the higher permeability of FD4 and HRP fluxes, we found a decreased expression of the TJs protein occludin, both on protein and mRNA level in IUGR neonatal piglets compared with NBW ones, This is inconsistent with a previous study showing that occludin protein expression was not different in the proximal SI between newborn IUGR and NBW piglets (Huygelen *et al.*, 2014), suggesting that a small fold change of mRNA may be accompanied by a different translational level outcome. In literature, it has been well described that the proinflammatory cytokine TNF-α could induce an increase in intestinal epithelial TJs permeability and contribute to intestinal inflammation (Ma *et al.*, 2004; Ye *et al.*, 2006). Surprisingly, compared to NBW littermates, the TNF-α expression was downregulated in IUGR piglets compared to NBW littermates, suggesting that the higher proximal SI permeability in IUGR piglets was not induced by an inflammatory response.

In the present study, IUGR piglets also showed an enhanced transcytosis (HRP flux) in the proximal SI as compared with age-matched NBW ones on DPW5. This higher transcytosis could be associated with either stress induced intestinal permeability increase or the presence of enterocyte lysosomal vacuoles. Notably, accompanied with this enhanced transcytosis, the intestinal absorptive capacity was also higher in the proximal SI at DPW5, the relationship between the enhanced transcytosis and better absorptive capacity needs further attention.

3 EARLY DEVELOPMENT OF INTESTINAL BARRIER FUNCTION IN IUGR AND NBW PIGLETS

3.1 Postnatal development of small intestinal barrier function

In the current study, during the **neonatal period**, proximal SI TJs permeability (FD4 flux) gradually increased from DPN3 to DPN19 in NBW piglets. In a study of Boudry *et al.* (2006), HRP and FD4 fluxes decreased in the jejunum, but increased in ileum with age in NBW piglets from DPN7 to 28. In another study from the same research group, it was shown that during the 28-day postnatal period, the jejunal FD4 flux of NBW piglets was highest on DPN21 and 28, followed by DPN0 and 3, and was lowest on DPN7 and 14 (De Quelen *et al.*, 2011). Although these results were from jejunum, they corroborate our finding that intestinal permeability increased at around 3-week of age. This age-dependent change of intestinal permeability could be associated with the protein level in the maternal milk. It has been proven that milk protein concentration has an effect on intestinal barrier function (Boudry *et al.*, 2011), partly by altering the expression and functions of TJs claudins (Kotler *et al.*, 2013). According to a milk production prediction model, with a litter size of twelve piglets, the protein content of maternal milk increases gradually until day 15, then decreases on day 20 of lactation (Hansen *et al.*, 2012), which corresponds to the altered intestinal permeability on DPN19 in this study. Considering that the maturation of GIT is particularly rapid during the perinatal period (Sangild *et al.*, 2002; van Elburg *et al.*, 2003). The postnatal development of intestinal mucosa is a dynamic process with profound tissue remodeling and modification of intestinal digestive and absorptive functions (Zabielski et al., 2008). Therefore, we cannot exclude the possibility that this increased permeability on DPN19 is due to the normal intestinal maturation process. Notably, this age-dependent increased intestinal permeability was not observed in IUGR piglets, which is consistent with the earlier finding that an alteration in jejunal permeability only takes place in NBW piglets but not in IUGR littermates (Boudry *et al.*, 2006; Boudry *et al.*, 2011).

About the TJs protein expression, the proximal SI *OCLN* mRNA level was higher on DPN3 and DPN8 compared with the level at birth in both IUGR and NBW piglets. Additionally, in IUGR piglets, the *OCLN* mRNA level decreased by 60% from DPN8 to DPN19. According to Huygelen *et al.* (2014), the proximal SI occludin expression was only higher on day 28 of age, but not on DPN3 and D10 of age. Since the fold-change of occludin mRNA level was less than 2-fold from DPN0 to DPN3 in the current study, it is common that this change could not be detected at the protein level. Balda *et al.* (1996) stated that when occludin reaches a certain level of expression, it has only sealing capacity to increase epithelial TEER, but can no longer regulate the paracellular permeability or activation of the channel forming (Balda *et al.*, 1996). It means that when the expression of occludin is low there are sufficient free binding sites on zonulins or other TJs proteins to interact with occludin; however, with increased occludin expression, the binding sites would become saturated, so that extra occludin would not be able to interact with submembrane cytoskeleton (Balda *et al.*, 1996). This characteristic of occludin may shed light on the discrepancy between developmental pattern of proximal SI permeability and occludin. Also, one has to bear in mind that occludin only functions to assembly the cytoskeleton and TJs proteins, it is possible that the regulation takes place on other TJs proteins, which needs further investigation.

3.2 Postweaning development of small intestinal barrier function

The digestive system of piglet can experience extensive structural and functional changes as an adaption to the shift from maternal milk to solid feed components during **postweaning period** (Zabielski *et al.*, 2008). Weaning-induced alterations in intestinal barrier function have been extensively documented in NBW piglets (Wijtten *et al.*, 2011). However, with regard to IUGR piglets, the information about postweaning intestinal development is fragmentary. The development of barrier function, particularly in the distal SI, has been well discussed in other literature, therefore, in this section, we will focus on the postnatal proximal SI barrier function development of IUGR and NBW piglets. On DPW5, the

increased proximal SI transcellular permeability of IUGR piglets was accompanied by reduced villus height and crypt depth. In contrast to the situation during the neonatal period, with the absence of changed proximal SI *OCLN* mRNA level (data not shown), the dynamic change of proximal permeability in IUGR piglets during postweaning period seemed to be associated with alteration in the intestinal mucosal architecture. As for NBW piglets, there was a reduction of villus height on DPW2, yet neither change in intestinal mucosal permeability nor crypt depth was observed when comparing to the preweaning level (DPN19). In a gut permeability assessment using *in vivo* SAT, the decreased mannitol absorption was attributed to the diminished absorptive area, indicated by a reduced villus height (Van Der Hulst *et al.*, 1998). However, in the current study, a definite correlation between the changed HRP fluxes with the villus atrophy could not be described because IUGR and NBW piglets showed different HRP fluxes alteration, but the same alteration of mucosal architecture from DPW0 to DPW2. During development, increased epithelial turnover can result in the immature cells reaching the absorptive area of the villi, and immature enterocytes are highly endocytic (Dunn, 1967; Wilson & Casanova, 2000; Boudry *et al.*, 2004; Söderholm *et al.*, 2004; Lu *et al.*, 2008). Because the crypt depth is related to the migration of immature enterocytes from crypts to villi (Nabuurs *et al.*, 1993), the sharply increased crypt depth on DPW2 in IUGR piglets might be an indication of the large presence of immature epithelial cells from the crypts to villi, leading to more active endocytosis. Additionally, the increased proximal SI HRP fluxes of IUGR piglets could not be related to weaning-induced intestinal local inflammation because mRNA level of *TNF-α* showed no alteration during the first five days postweaning. In IUGR piglets, the *TNF-α* gene expression was only upregulated on DPW12 and DPW28. Thus, we can speculate that a TNF-α induced alteration in intestinal permeability happened after DPW12 in IUGR piglets.

It has been proven that weaning triggers intestinal inflammation in pigs is associated with postweaning anorexia (McCracken *et al.*, 1999; Pie *et al.*, 2004). In Chapter IV, it was shown that during the first

5 days postweaning, IUGR piglets had a higher feed intake than the NBW ones, indicating that weaning induced aneroxia may be less prominent in IUGR piglets. Meanwhile, the change in diet composition, the increased exposure to enteric pathogens, and stress could has influence on the intestinal structure and function during a later stage of the postweaning period in IUGR piglets (Moeser *et al.*, 2012). Taken together, the postweaning barrier function development pattern in the proximal SI was different between IUGR and NBW piglets, especially the transcellular pathway. Weaning-induced reduced villus height, accompanied by increased intestinal permeability was observed on DPW5 in NBW piglets, and this process might be postponed in IUGR piglets.

4 EFFECT OF IUGR ON INTESTINAL REDOX STATUS AND REDOX-SENSITIVE GENE EXPRESSION IN EARLY LIFE OF PIGLETS

4.1 Effect of IUGR on intestinal redox-sensitive gene expression of piglets

On transcriptional level, we have investigated the expression of several redox-sensitive genes by RT-qPCR. The protein expression of some interesting ones was verified by western blotting. At birth, compared to the NBW piglets, the IUGR newborns showed significant downregulation of antioxidant genes including *HMOX1, CAT, TXNRD1*, and *PPARG*. Birth itself is a hyperoxic challenge, so the process of labor is associated with oxidative stress in the newborn (Vlessis & Mela-Riker, 1989; Friel *et al.*, 2004; Menon, 2014). In response to this challenge, biochemical defenses including antioxidant enzymes, evolved to protect cellular constituents from free radicals (Vlessis & Mela-Riker, 1989; Friel *et al.*, 2004). At birth, the downregulated antioxidant genes in IUGR piglets compared with the NBW ones, indicates that IUGR piglets might have a reduced antioxidant resistance to the perinatal oxidative challenge. Additionally, oxidative stress can activate a variety of transcription factors, such as NF-κB, Nrf2, and PPARγ, leading to the expression of different genes, including those of growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules and anti-inflammatory molecules (Reuter *et al.*, 2010; Menon, 2014). ROS are important second messengers of signaling pathways

stimulated by TNF-α, such as NF-κB (Schutze *et al.*, 1995). Suppression of TNF-α was shown to downregulate the expression an active NF-κB and inhibit proliferation of cells (Giri & Aggarwal, 1998). In the current study, compared with the NBW littermate, the lower expression of TNF-α both on mRNA and protein level in the newborn IUGR piglets might be an implication of downregulated NF-κB expression, which could further result in the downregulation of antioxidant genes such as *TXNRD1*. On the other hand, given that inhibition of NF-κB activation can block cell proliferation (Rath & Aggarwal, 2001), the downregulation of NF-κB targeted genes from this study is in line with the previous data showing lower ileum cell proliferation in IUGR neonatal piglets, compared to the NBW ones (D'Inca *et al.*, 2010b). Similarly, a recent study reported decreased cellular proliferation, as well as a reduction in goblet cells and Paneth cells in IUGR mouse (Fung *et al.*, 2016). It has been stated that in intestinal cells, a genetic program is maintained that allows the cell to proliferate only when being subjected to a stimulus such as an antigen, a mitogen, or increased oxidant, or a combination of these (Flores & McCord, 1997). Therefore, the lower expression of TNF-α in IUGR piglets at birth compared to the NBW ones could contribute to the failure to initiate the epithelial cell proliferation and differentiation process, thus delaying the intestinal development in IUGR newborns.

PPARγ is a lipid sensing transcriptional factor that is involved in embryonic development, modulating energy metabolism, lipid storage/transport, as well as inflammation (Nunn *et al.*, 2007). Inhibition of PPARγ activity by TNF-α is involved in the pathogenesis of insulin resistance and inflammation (Ye, 2008). In turn, PPARγ can exert an anti-inflammatory response by inhibiting TNF-α induced NF-κB transcriptional activity (Remels *et al.*, 2009). In IUGR rat newborns, downregulated *PPARG* expression was also found in liver, and it was concluded that the hepatic dysregulation of *PPARG* may contribute to inflammation in IUGR offspring (Magee *et al.*, 2008). However, in the present study, the simultaneous occurrence of lower expression of the *TNF-α* and *PPARG* mRNA level in the

proximal SI on DPN0 in IUGR piglets, as compared to the NBW piglets, suggests that regulation of the intestinal *TNF-α* level is rather through a ROS-related signaling pathway than an inflammation related pathway. Thus, the TNF-α may play a central role redox-sensitive signaling pathway like NF-κB, and the redox-sensitive signaling ways in IUGR piglets worthy further attention.

Similarly, downregulated antioxidant genes were also observed at DPN19 of age in IUGR piglets compared to the age-matched NBW piglets. In contrast to the observation that TNF-α was downregulated in IUGR neonates, IUGR piglets showed significantly higher *TNF-α* gene expression on DPN19 of age compared to the NBW ones, suggesting an inflammatory response in the proximal SI. More evidence is provided by the higher expression of *VNN1*, an epithelial stress sensor. By antagonizing PPARγ, VNN1 licenses the production of inflammatory mediators in intestinal epithelial cells, and its deficiency is associated with increased GSH concentration (Berruyer *et al.*, 2004; Berruyer *et al.*, 2006). The higher expression of *VNN1* and *TNF-α*, but lower expression of antioxidant genes and *PPARG* indicates that IUGR negatively affects the intestinal defense to stress.

During the **postweaning period**, several antioxidant genes including *GPX1, PPARG and TXNRD1* expressions were downregulated in IUGR piglets compared to NBW ones on DPW5. Similar to the observation at birth, the downregulation of these protective genes was accompanied by increased paracellular permeability but lower *TNF-α* expression. Therefore, the results of gene expression on DPW5 may suggest that IUGR still affect the NF-κB mediated intestinal cell proliferation after weaning, which might be a reason for the retarded growth of SI.

4.2 Effect of IUGR on intestinal GSSG/GSH ratio and MDA content of piglets

GSH and its oxidized form GSSG are of crucial importance for cellular function (Brigelius, 1985). The intestinal GSSG/GSH ratio has been widely used as an indicator of the redox status of the intestinal mucosa (Aw, 1999). In the current study, we did not find any difference in GSSG/GSH

ratios between IUGR and NBW piglets at any time points. Only a higher proximal GSH concentration was found in IUGR piglets on DPN19, compared to the age-matched NBW littermates. Besides, this difference was only observed in the piglets sampled in the postweaning experiment, suggesting a variation among litters. It is important to note that large increases in GSSG/GSH ratio are usually a sign of oxidative stress causing toxicity, rather than signaling associated with redox biology (Morgan *et al.*, 2011). Also, it is possible that the changes in ROS required for signaling may be not be high enough to cause significant changes in the intracellular GSSG/GSH ratio (Murphy, 2012). The unaffected GSSG/GSH ratio was in line with the absence of a difference in intestinal MDA content in intestinal mucosa in IUGR piglets compared with the NBW ones at each time point in the early life. MDA is the principal and most studied product of lipid peroxidation (Del Rio *et al.*, 2005). Mucosal GSH plays an important role in controlling lipid peroxide content. However, results from this study suggest that IUGR can not affect the lipid peroxidation process through regulating the GSSG/GSH ratio.

It has been shown that in VNN1 deficient mice, the lack of detectable tissue cysteamine is associated with an enhanced GCL activity, leading to elevated endogenous glutathione (GSH) stores in tissues (Berruyer *et al.*, 2004). In contrast with this report, we found higher GSH concentration as well as higher *VNN1* expression in proximal intestine of IUGR piglets on DPN19. This indicates that the *in vivo* regulation of GSH concentration is complex.

5 REDOX-RELATED CHANGES DURING EARLY LIFE IN IUGR AND NBW PIGLETS

In this study, **similar changes** in the proximal SI of both IUGR and NBW piglets were as follows,

1) Decreased MDA concentration from DPN0 to DPN3. In the current study, all the newborn piglets in this study were allowed to have colostrum intake. Therefore, the higher MDA at birth could evidence that there is an imbalance between the capacity to oxidized lipid and to remove the lipid peroxidation product in the intestine of newborn piglets (Le Dividich *et al.*, 1997).

Although the MDA concentration decreased quickly on DPN3, the effect of colostrum intake of the functional change of newborn piglets needs further attention.

2) Increased *OCLN* mRNA level from DPN0 to DPN3. Occludin is a redox-sensitive marker of TJs. The increased *OCLN* mRNA level indicates that sealing the paracellular pathway might also contribute to the process of "gut closure". Also, since occludin has been widely documented to be altered due to changes in its environmental redox balance on molecular level (Blasig *et al.*, 2011), this increased *OCLN* mRNA may offer a link between intestinal barrier function and redox status or redox signaling.
3) HO has antioxidant function. Meanwhile, it can inhibit NF-κB activation, a gradual reduction of *HMOX1* expression during the whole neonatal period could be an indication of protective mechanism to NF-κB.
4) Decreased GSSG/GSH ratio from DPW0 to DPW2. Comparing to the preweaning level, the intestinal mucosa was in a less oxidative state during the whole postweaning period. Whether this decreased ratio resulted from weaning induced anorexia or stress needs further investigation.
5) Increased crypt depth from DPW2 to DPW5, suggesting that adaptation of SI epithelial mucosa to the change in feed composition induced crypt cell proliferation.

Several particular redox-related changes in the proximal SI of **IUGR piglets** were noticed.

1) GSH concentration reduction in the proximal SI (from DPN0 to DPN3, and from DPN8 to DPN19) was always accompanied by a decreased MDA level, demonstrating that the intestinal GSH metabolism in IUGR piglets is related with intestinal lipid peroxidation.
2) Increased *SOD1* mRNA level was noticed from DPN0 to DPN3, and from before weaning to DPW2. A recent study indicated that SOD1 is essential for growth factor signaling (Juarez *et al.*, 2008). Since the two time points when SOD1 were upregulated corresponds to the main

change taking place in piglets, the effect of SOD1 during the early life the animal needs further attention.

3) The increase of *TNF-α* and some other antioxidant genes may indicate an upregulation of NF-κB activity, which could further initiate the start the intestinal cell proliferation. This is further supported by the increased proliferating cell nuclear antigen (PCNA) gene expression in the ileum of IUGR piglets on DPN2 in a previous study (D'Inca *et al.*, 2010b).

6 GSH METABOLISM IS AFFECTED BY IUGR UNDER OXIDATIVE STRESS

In Chapter IV, we introduced oxidized fat in the diet of weanling piglets to establish an oxidative challenge model, to address the effect of IUGR on GSH metabolism under oxidative stress. In line with the results obtained on DPW5 in Chapter III, we found no change in GSH/GSSG redox couple on day 5 in the oxidative challenge trial. This could be an implication that during the acute phase after weaning, weaning stress is more effective in inducing oxidative stress than the challenge of oxidized fat. However, in the current Ph.D. study, most of the feed intake was recorded based on pen level with limited replicates per pen (4 or 6), how these results can represent the practical industry is debatable. Additionally, the ADFI of OFNBW piglets were lower than OFCON piglets during day 5 to 26 of the experimental period, nevertheless, the MDA content in the proximal SI mucosa was higher in OFNBW piglets than the OFCON ones, indicating the intake of oxidized fat may induce accumulation of MDA in the SI. The lower MDA, as well as GSH concentration in the IUGR piglets could be due to the lower intake of oxidized fat as compared to the OFNBW piglets, however, the GSSG/GSH ratio was still higher in the proximal SI mucosa of OFIUGR piglets, suggesting the more oxidized state of the proximal SI is irrespective of feed intake level.

Mucosal GSH plays an important role in controlling lipid peroxide content. Under normal condition, we found that the proximal SI GSH concentration reduction (from DPN0 to DPN3, and from DPN8

to DPN19) was always accompanied by a decreased MDA level, indicating that the intestinal GSH metabolism in IUGR piglets is related with intestinal lipid peroxidation. Similar to the results we obtained on DPW5 in Chapter III, in the oxidative challenge trail, we found that on day 5, no change was found in the intestinal GSH/GSSG redox couple, which is likely due to the extremely low feed caused intake caused by weaning anorexia. On day 28, although the feed intake of OFIUGR piglets was lower than the OFNBW ones, numerous differences were found in GSH metabolism in the proximal SI mucosa, plasma and liver, indicating a successful oxidative challenge from long-term OF intervention. Also, the results in the oxidative challenge trail further supported that GSH metabolism is related with lipid peroxidation. To note, this effect was only observed in the proximal SI, which is likely due to lipid digestion taken place in this site of SI, or that hepatic GSH export has more effect on proximal than the distal SI. Interestingly, the lower intestinal MDA concentration in IUGR piglets as compared to NBW piglets is accompanied with a lower GSH concentration, whereas higher MDA content and lower GSH concentration in plasma, and similar MDA with higher GSH concentration in liver were presented. These results suggest that the intestinal GSH metabolism is under different regulation mechanism than liver and circulatory system.

7 CONCLUSIONS

In this study, we investigated the intestinal barrier function, redox status and redox-sensitive molecular consequences of IUGR. We have demonstrated that IUGR could affect the intestinal barrier function during the neonatal period (day 0 to day 19 of age) through downregulating the TJs protein OCLN both on transcriptional and translation level. Some antioxidant genes and the pro-inflammatory cytokine TNF-α were also affected, suggesting a potential role of redox-sensitive signaling in driving IUGR intestinal development during the neonatal period. After weaning, the difference of intestinal barrier function between IUGR and NBW piglets seems to be more associated with the difference in intestinal mucosal architecture. The weaning-induced barrier function loss is also accompanied by the

TNF-α expression, and this response might be delayed in IUGR piglets. Finally, we have demonstrated that under normal physiological conditions, IUGR does not affect the intestinal redox status through regulating the mucosal GSH/GSSG redox couple. Furthermore, after chronic oxidative challenge, weaned IUGR piglets showed lower intestinal GSH concentration, but higher GSSG/GSH ratio, indicating that the intestine was in a more oxidative state.

8 FUTURE PERSPECTIVES

This current thesis has shed some light on the effect of IUGR on the intestinal barrier function and antioxidant systems. However, considering the sophisticated physiological function of the small intestine, and the discrepancy in the effect of IUGR on intestinal function and redox status, we propose the following aspect for further research:

- Additional oxidative stress marker should be selected to address whether newborn piglets are suffering oxidative stress. Given the fact that difference of intestinal MDA is absence during the early development of SI, it would be interesting to identify a sensitive oxidative stress marker to illustrate the link between newborns and oxidative stress.
- Results of this study showed an effect of IUGR on the regulation of some antioxidant genes, yet the factors driving this alteration of genes expression remain unknown. ROS have been shown to serve as critical second messengers in multiple signal transaction pathways stimulated by pro-inflammatory cytokines and growth factors (Jones *et al.*, 2012). Therefore, understanding the ROS level in IUGR newborns might be interesting to find out the initial reason of these transcriptional differences induced by IUGR.
- The relationship between intestinal cell proliferation and redox-sensitive signaling in IUGR piglets worth further investigation. It has been shown that the immature intestine of IUGR piglets could be attributed to a lower intestinal proliferation (D'Inca *et al.*, 2010b). Based on

gene expression data in the current study, we propose that this lower intestinal proliferation is related to the ROS-induced NF-κB activation. However, a direct link between intestinal cell proliferation and this redox-sensitive pathway has not been addressed. The hypothesis that IUGR could affect intestinal cell proliferation by suppressing NF-κB pathway in piglets is highly speculative and remains to be tested, but the simultaneous downregulation of antioxidant genes and inhibited cell proliferation during the first days of the life of IUGR piglets, appear to support such a hypothesis. The better understanding of the signaling pathways could provide further information on the effects of nutrition intervention strategies in these low birth weight piglets.

- In this study, the largest differences in intestinal mucosal permeability were found in the proximal site. Sampling at 5% and 75% of the SI refers to duodenum/proximal jejunum and distal jejunum, respectively. In the duodenum, chyme from pancreatic juice and bile is mixed. Therefore, the fluid from liver may has more influence on the redox status of the proximal SI than the distal SI. Also, the proportions of absorptive, goblet and enteroendocrine cells differ in different segments of the SI. The ratio of goblet to absorptive cells is higher in the distal SI. In addition, several studies have shown that the development of the intestinal epithelium is site specific (Jolma *et al.*, 1980; Drozdowski *et al.*, 2010). Thus, cells responsible for this differential development needs further attention.

SUMMARY

In pig production, the genetic selection of highly productive sows has resulted in a large amount of IUGR piglets. The poor health status of IUGR piglets is presumably due to digestive problems, because it was found that 80% of IUGR piglets that subsequently died, manifested digestive disorders. As the largest and most important barrier against the external environment, the intestinal epithelium plays important role in the absorption of nutrients, electrolytes, and water while maintaining an adequate defense against intraluminal antigens, and bacteria. There has been increasing recognition of an association between disrupted intestinal barrier function and the pathogenesis of intestinal dysfunctions. Also, the intestinal epithelium is exposed to reactive oxygen metabolites from multiple luminal and systemic sources. Therefore, it possesses several antioxidant systems to maintain a proper redox status. As for piglets, there is mounting evidence that IUGR has a transient effect on the intestinal barrier function at birth, as well as a long-term effect on some intestinal redox-sensitive parameters during the postnatal period. However, limited and conflicting results have been reported in this respect for the postnatal period. We also noticed that most literature focused on investigating the effect of IUGR on the distal SI without paying enough attention to the proximal site. Additionally, information about the redox status of IUGR piglets during early life is fragmental. In **Chapter I**, a literature review focused on general knowledge about IUGR and its importance in pig production. The intestinal mucosal physiology and early development in pigs, as well as the role of oxidative stress in intestinal physiology were presented.

In **Chapter II** and **Chapter III**, the effects of IUGR on the intestinal barrier function and redox-sensitive parameters were investigated during the neonatal (preweaning) and postweaning period. The experiment was conducted on IUGR and NBW littermates in a 47-day period from birth on and with weaning at 19 days of age. Samples were taken at different time points in the neonatal (DPN0, DPN3,

DPN8 and DPN19) and postweaning period (DPW2, DPW5, DPW8, DPW12 and DPW28). On every sampling time point, the proximal and distal SI mucosa from IUGR and NBW littermates was collected, and intestinal barrier function was assessed by *ex vivo* permeability of FD4 and HRP in Ussing chamber. The GSSG/GSH ratio and MDA content were measured to evaluate the redox status of the intestinal mucosa. On transcriptional level, the expression of the TJ protein occludin, the proinflammatory cytokine TNF-α and several redox-sensitive genes were qualified by RT-qPCR.

During the neonatal period, IUGR piglets exhibited higher intestinal FD4 and HRP fluxes in the proximal SI on DPN0 and DPN3, compared to the age-matched NBW ones, suggesting a more active endocytosis and disrupted intestinal TJs function. The histomorphological analysis showed that the mucosal architecture could not be associated with this higher mucosal permeability, whereas the protein and mRNA level of TJs protein OCLN were lower in IUGR piglets on DPN0. However, on DPN3, the OCLN expression was similar in IUGR and NBW piglets, suggesting that the higher intestinal mucosal permeability on DPN3 could not be attributed to OCLN. In the distal SI, only a higher HRP flux was observed, suggesting a more active endocytosis in the enterocytes, which could be a sign of more presence of immature cells.

Interestingly, we found that the expression of the proinflammatory cytokine TNF-α was lower in IUGR piglets at birth, compared to NBW littermates. This finding may have two implications. First, the higher intestinal permeability in IUGR piglets is not induced by TNF-α; second, some TNF-α induced signaling pathways were not activated in IUGR piglets at birth. For example, the transcriptional factor *PPARG*, as well as *HMOX1* was down regulated, probably from a downregulated Nrf2 pathway. Also, the downregulated *CAT* and *TXNRD1* could be the result of downregulated NF-κB pathway. The activity of NF-κB could be affected by ROS levels in the cell, and inhibition of the NF-κB pathway could have an effect on the intestinal cell proliferation. Thus, these downregulated

genes could mean that the signaling pathways necessary for initiating intestinal cell proliferation were not activated, leading to a retarded intestinal maturation. However, the NF-κB activity was not investigated in this study, and the relationship between ROS affecting the NF-κB pathway and intestinal cell proliferation worths further attention.

During the postweaning period, we noticed a higher HRP flux on DPW5 in weaners born with IUGR than NBW ones. This higher endocytosis could be associated with the better intestinal absorption as indicated by a higher V/C ratio in IUGR piglets. Moreover, on DPW5, we noticed a lower expression of intestinal TNF-α and antioxidant genes such as *PPARG, TXNRD1* and *GPX1*. A possible reason for this transient difference on DPW5 is that the weaning stress-induced villus atrophy happened from DPW2 to DPW5 in NBW piglets, but was postponed to DPW5 to DPW12in IUGR piglets.

Throughout the first 7-weeks of life, the piglets born with IUGR have shown lower body weight than the NBW littermates on each time point, indicating a long-term effect of IUGR on reduced growth performance in piglets. As for the intestinal barrier function, in agreement with other studies, the current results showed that IUGR has a transient effect on intestinal barrier function in newborn piglets, and this effect disappears with age. Our findings also provide a potential link between the reduced intestinal cell proliferation induced retarded growth and intestinal ROS-induced signaling pathway. Surprisingly, we noticed no difference between IUGR and NBW piglets for the GSSG/GSH ratio and MDA concentration throughout the 7-week experimental period, indicating that the intestinal redox status is not affected by IUGR.

During the first two studies, the nature of oxidative challenge was not well defined. In **Chapter IV**, we introduced 5% oxidized linseed oil in the diet of weanling piglets to check the redox status of IUGR and NBW littermates under chronic oxidative challenge. After 5 or 28 days, the GSSG/GSH ratio, as well as activity and transcription of major enzymes involved in the GSH redox cycle were evaluated in

the liver, proximal and distal site of SI and blood. The higher GSH concentration in plasma in IUGR piglets compared to the NBW littermates on day 5 of the experimental period, was probably due to the weaning anorexia. On day 28, the higher GSSG/GSH ratio and lower storage of GSH in the proximal SI of IUGR piglets indicate that the epithelial cells were in the oxidized state, although the feed intake was lower for IUGR piglets than for NBW piglets. Moreover, it seems that the SI of IUGR piglets allows a greater entry of MDA to circulation. Finally, the regulation pattern of redox-sensitive enzymes involved in GSH redox cycle at the transcriptional level was different between IUGR and NBW piglets when under oxidative challenge.

In Chapter V, the results from the preweaning and postweaning experiments are compared and discussed. In this respect, we found some intestinal disorder induced by IUGR could last till postweaning period, e.g., the increased intestinal transcytosis on DPW5 of IUGR piglets is similar to the observation on DPN3. Finally, the limitations of this Ph.D. research and future research directions are presented.

Altogether, the findings of the current Ph.D. research have demonstrated a delayed SI maturation in IUGR piglets. The SI of IUGR showed a lack of adaptation to the nutrient shift both at birth and at weaning. These results can be explained, at least in part, by the attenuated redox-sensitive signaling pathway in the SI of IUGR piglets. However, more studies are needed to clarify the potential association between delayed intestinal growth and redox-sensitive signaling pathways.

SAMENVATTING

In de varkenshouderij heeft de genetische selectie van hoogproductieve zeugen geleid tot een groot aantal IUGR biggen. De slechte gezondheidstoestand van IUGR biggen is vermoedelijk te wijten aan spijsverteringsproblemen, omdat 80% van de gestorven IUGR biggen voorafgaand symptomen van spijsverteringsstoornissen vertonen. Aangezien het de grootste en meest belangrijkste barrière is tegen de buitenomgeving, speelt het intestinale epithelium een belangrijke rol in de absorptie van nutriënten, elektrolyten en water, terwijl ze eveneens een adequate verdediging vormt tegenover intra-luminale antigenen en bacteriën. Er komt steeds meer erkenning voor een associatie tussen een verstoorde functie van de intestinale barrière en de pathogenese van intestinale stoornissen. Het intestinale epithelium is eveneens blootgesteld aan reactieve zuurstofradicalen van zowel luminale als systemische oorsprong. Hierdoor bezit het verscheidene antioxidant systemen om een goede redox status te behouden. Bij biggen is er steeds meer bewijs dat IUGR zowel een kortdurend effect heeft op de intestinale barrière functie bij de geboorte, als een langdurend effect op bepaalde intestinale redox-gevoelige parameters gedurende de postnatale periode. In dit opzicht werden echter beperkte en tegenstrijdige resultaten gerapporteerd voor de postnatale periode. We merkten ook dat de meeste literatuur zich voornamelijk richtte op het effect van IUGR op de distale dunne darm, zonder genoeg rekening te houden met het proximale segment. Bijkomend is de informatie over de redox status van IUGR biggen in de vroege levensfase fragmentarisch. In Hoofdstuk I richtte een literatuurstudie zich op de algemene kennis over IUGR en het belang in de varkenshouderij. Zowel de intestinale mucosale fysiologie en vroege ontwikkeling als de rol van oxidatieve stress in de intestinale fysiologie werden besproken voor biggen.

In Hoofdstuk II en Hoofdstuk III werden de effecten van IUGR op de intestinale barrière functie en redox-gevoelige parameters onderzocht tijdens de neonatale periode (voor spenen) en de periode na het spenen. Het experiment werd uitgevoerd op IUGR en NBW nestgenoten in een periode van 47 dagen

vanaf de geboorte, en de biggen werden gespeend op een leeftijd van 19 dagen. Stalen werden genomen op verschillende tijdstippen in de neonatale periode (DPN0, DPN3, DPN8 en DPN19) en in de periode na het spenen (DPW2, DPW5, DPW8, DPW12 en DPW28). Op elk tijdstip werden de proximale en distale dunne darmmucosa van IUGR en NBW nestgenoten verzameld, en werd de ex vivo permeabiliteit van FD4 en HRP beoordeeld in Ussing kamers. De GSSG/GSH ratio en de MDA concentraties werden gemeten om de redox status van de intestinale mucosa te beoordelen. Op het niveau van transcriptie werd de expressie van het TJ proteïne occludine, het pro-inflammatoire cytokine TNF-α en verscheidene redox-gevoelige genen semi-kwantitatief gemeten via RT-qPCR.

Tijdens de neonatale periode vertoonden IUGR biggen een hogere intestinale FD4 en HRP flux in de proximale dunne darm op DPN0 en DPN3, vergeleken met de NBW biggen van dezelfde leeftijd, hetgeen een meer actieve endocytose en verstoorde intestinale functie van de TJs suggereert. Histomorfologisch onderzoek toonde aan dat de mucosale architectuur niet geassocieerd was met deze hogere mucosale permeabiliteit, terwijl de proteïne en mRNA niveaus van het TJ proteïne OCLN lager waren in de IUGR biggen op DPN0. Op DPN3 was de expressie van OCLN echter gelijkaardig in IUGR en NBW biggen, suggererend dat de hogere intestinale mucosale permeabiliteit op DPN3 niet kon toegeschreven worden aan OCLN. In de distale dunne darm werd enkel een hogere HRP flux geobserveerd, hetgeen een meer actieve endocytose in de enterocyten suggereert, wat een teken kan zijn van een hogere aanwezigheid van immature cellen.

Tijdens de periode na het spenen, observeerden we een hogere HRP flux bij DPW5 in gespeende biggen met IUGR, vergeleken met NBW. Deze hogere endocytose kan geassocieerd worden met de betere intestinale absorptie, zoals aangegeven door een hogere V/C ratio in IUGR biggen. Bovendien observeerden we op DPW5 een lagere expressie van intestinaal *TNF-α* en antioxidant genen zoals *PPARG, TXNRD1* en *GPX1*. Een mogelijke verklaring voor dit kortdurend verschil op DPW5 is dat

de speenstress-geïnduceerde villus atrofie gebeurde van DPW2 tot DPW5 in NBW biggen, maar uitgesteld was in de IUGR biggen van DPW5 tot DPW12.

Doorheen de eerste 7 levensweken toonden de biggen geboren met IUGR een lager lichaamsgewicht dan de NBW nestgenoten op elk tijdstip, wat duidt op een langdurend effect van IUGR op verminderde groei in biggen. Wat betreft de intestinale barrière functie, toonden de huidige resultaten in overeenstemming met andere studies, dat IUGR een kortdurend effect heeft op de intestinale barrière functie in nieuwgeboren biggen, en dit effect verdwijnt met de leeftijd. Onze bevindingen bieden ook een mogelijke link tussen de vertraagde groei door een gereduceerde intestinale celproliferatie, en intestinale ROS-geïnduceerde signaalroutes. Een verrassende observatie was dat er geen verschil was tussen IUGR en NBW biggen voor de GSSG/GSH ratio en MDA concentraties doorheen het 7 weken durende experiment, wat erop wijst dat de intestinale redox status niet beïnvloed is door IUGR.

Tijdens de eerste twee studies was de aard van de oxidatieve challenge niet goed gedefinieerd. In Hoofstuk IV introduceerden we 5% geoxideerde lijnzaadolie in het dieet van gespeende biggen om de redox status van IUGR en NBW nestgenoten te onderzoeken bij blootstelling aan een chronische oxidatieve challenge. Na 5 of 28 dagen werden zowel de GSSG/GSH ratio als de activiteit en transcriptie van de belangrijkste enzymen betrokken in de GSH redox cyclus onderzocht in de lever, de proximale en distale sectie van de dunne darm en in het bloed. De hogere GSH concentraties in plasma van IUGR biggen, vergeleken met NBW nestgenoten op dag 5 van de experimentele periode, waren waarschijnlijk veroorzaakt door speen anorexia. De hogere GSSG/GSH ratio en de lagere GSH concentraties in het proximale segment van IUGR biggen op dag 28 wijzen erop dat de epitheliale cellen zich in een geoxideerde status bevonden, alhoewel de voederopname lager was voor de IUGR biggen vergeleken met de NBW biggen. Bovendien bleek dat de dunne darm van IUGR biggen een grotere absorptie van MDA in de algemene circulatie toeliet. Tot slot was de regulatie van redox-

gevoelige enzymen betrokken in de GSH redox cyclus op het transcriptie niveau verschillend tussen IUGR en NBW biggen bij blootstelling aan een oxidatieve challenge.

In Hoofdstuk V werden de resultaten van de periodes voor en na spenen overheen de verschillende experimenten vergeleken en gediscussieerd. In dit opzicht vonden we dat sommige intestinale stoornissen geïnduceerd door IUGR kunnen blijven duren tot na het spenen, bv. de verhoogde intestinale transcytose op DPW5 van de IUGR biggen is gelijkaardig als de observatie op DPN3. Tot slot werden de beperkingen van dit doctoraatsonderzoek en verdere onderzoeksrichtingen besproken.

Samengevat toonden de bevindingen van deze doctoraatsthesis een vertraagde ontwikkeling van de dunne darm bij IUGR biggen aan. De dunne darm van IUGR biggen vertoonde een aanpassingsgebrek aan de shift in nutriënten zowel bij de geboorte als tijdens het spenen. Deze resultaten kunnen gedeeltelijk verklaard worden door een verzwakte redox-gevoelige signaalroute in de dunne darm van IUGR biggen. Meer studies zijn echter noodzakelijk om de mogelijke associatie tussen de uitgestelde intestinale ontwikkeling en de redox-gevoelige signaalroutes te verklaren.

REFERENCES

A

Allen, W. R., Wilsher, S., Turnbull, C., Stewart, F., Ousey, J., Rossdale, P. D., & Fowden, A. L. (2002). Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. *Reproduction, 123*(3), 445-453

Ames, B. N. (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science, 221*(4617), 1256-1264

Ananth, C. V., & Vintzileos, A. M. (2009). Distinguishing pathological from constitutional small for gestational age births in population-based studies. *Early Human Development, 85*(10), 653-658

Ashworth, C. J., Finch, A. M., Page, K. R., Nwagwu, M. O., & McArdle, H. J. (2001). Causes and consequences of fetal growth retardation in pigs. *Reproduction Supplement, 58*, 233-246

Atisook, K., & Madara, J. L. (1991). An Oligopeptide Permeates Intestinal Tight Junctions at Glucose-Elicited Dilatations - Implications for Oligopeptide Absorption. *Gastroenterology, 100*(3), 719-724

Aucott, S. W., Donohue, P. K., & Northington, F. J. (2004). Increased morbidity in severe early intrauterine growth restriction. *Journal of Perinatology, 24*(7), 435-440

Aumaitre, A., & Corring, T. (1978). Development of digestive enzymes in the piglet from birth to 8 weeks. II. Intestine and intestinal disaccharidases. *Nutrition and Metabolism, 22*(4), 244-255

Aw, T. Y. (1994). Biliary glutathione promotes the mucosal metabolism of luminal peroxidized lipids by rat small intestine in vivo. *Journal of Clinical Investigation, 94*(3), 1218-1225

Aw, T. Y. (1999). Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. *American Journal of Clinical Nutrition, 70*(4), 557-565

Aw, T. Y. (2005). Intestinal glutathione: determinant of mucosal peroxide transport, metabolism, and oxidative susceptibility. *Toxicology and Applied Pharmacology, 204*(3), 320-328

Aw, T. Y., Williams, M. W., & Gray, L. (1992). Absorption and lymphatic transport of peroxidized lipids by rat small intestine in vivo: role of mucosal GSH. *American Journal of Physiology, 262*(1 Pt 1), G99-106

Awada, M., Soulage, C. O., Meynier, A., Debard, C., Plaisancie, P., Benoit, B., Picard, G., Loizon, E., Chauvin, M. A., Estienne, M., Peretti, N., Guichardant, M., Lagarde, M., Genot, C., & Michalski, M. C. (2012). Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *Journal of Lipid Research, 53*(10), 2069-2080

B

Balda, M. S., Whitney, J. A., Flores, C., Gonzalez, S., Cereijido, M., & Matter, K. (1996). Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the

apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *Molecular Biology of the Cell, 7*, 3534-3534

Balogh, L. M., Le Trong, I., Kripps, K. A., Shireman, L. M., Stenkamp, R. E., Zhang, W., Mannervik, B., & Atkins, W. M. (2010). Substrate specificity combined with stereopromiscuity in glutathione transferase A4-4-dependent metabolism of 4-hydroxynonenal. *Biochemistry, 49*(7), 1541-1548

Banning, A., & Brigelius-Flohé, R. (2005). NF-kappaB, Nrf2, and HO-1 interplay in redox-regulated VCAM-1 expression. *Antioxidants & Redox Signaling, 7*(7-8), 889-899

Bauer, E., Metzler-Zebeli, B. U., Verstegen, M. W. A., & Mosenthin, R. (2011). Intestinal gene expression in pigs: effects of reduced feed intake during weaning and potential impact of dietary components. *Nutrition Research Reviews, 24*(02), 155-175

Bauer, R., Walter, B., Hoppe, A., Gaser, E., Lampe, V., Kauf, E., & Zwiener, U. (1998a). Body weight distribution and organ size in newborn swine (sus scrofa domestica) -- a study describing an animal model for asymmetrical intrauterine growth retardation. *Experimental and Toxicologic Pathology, 50*(1), 59-65

Bauer, R., Walter, B., Hoppe, A., Gaser, E., Lampe, V., Kauf, E., & Zwiener, U. (1998b). Body weight distribution and organ size in newborn swine (sus scrofa domestica) — A study describing an animal model for asymmetrical intrauterine growth retardation. *Experimental and Toxicologic Pathology, 50*(1), 59-65

Berruyer, C., Martin, F. M., Castellano, R., Macone, A., Malergue, F., Garrido-Urbani, S., Millet, V., Imbert, J., Dupre, S., Pitari, G., Naquet, P., & Galland, F. (2004). Vanin-1-/- mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Molecular and Cellular Biology, 24*(16), 7214-7224

Berruyer, C., Pouyet, L., Millet, V., Martin, F. M., LeGoffic, A., Canonici, A., Garcia, S., Bagnis, C., Naquet, P., & Galland, F. (2006). Vanin-1 licenses inflammatory mediator production by gut epithelial cells and controls colitis by antagonizing peroxisome proliferator-activated receptor gamma activity. *Journal of Experimental Medicine, 203*(13), 2817-2827

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews, 94*(2), 329-354

Biotek.com. An Introduction to Reactive Oxygen Species - Measurement of ROS in Cells. Retrieved from http://www.biotek.com/resources/articles/reactive-oxygen-species.html

Biri, A., Bozkurt, N., Turp, A., Kavutcu, M., Himmetoglu, O., & Durak, I. (2007). Role of oxidative stress in intrauterine growth restriction. *Gynecologic and Obstetric Investigation, 64*(4), 187-192

Blanquicett, C., Kang, B. Y., Ritzenthaler, J. D., Jones, D. P., & Hart, C. M. (2010). Oxidative stress modulates PPAR gamma in vascular endothelial cells. *Free Radical Biology and Medicine, 48*(12), 1618-1625

Blasig, I. E., Bellmann, C., Cording, J., Del Vecchio, G., Zwanziger, D., Huber, O., & Haseloff, R. F. (2011). Occludin protein family: oxidative stress and reducing conditions. *Antioxid Redox Signal, 15*(5), 1195-1219

Blikslager, A. T., Moeser, A. J., Gookin, J. L., Jones, S. L., & Odle, J. (2007). Restoration of barrier function in injured intestinal mucosa. *Physiological Reviews, 87*(2), 545-564

Boudry, G. (2005). The Ussing chamber technique to evaluate alternatives to in-feed antibiotics for young pigs. *Animal Research, 54*(3), 219-230

Boudry, G., Lalles, J. P., Malbert, C. H., Bobillier, E., & Seve, B. (2002). Diet-related adaptation of the small intestine at weaning in pigs is functional rather than structural. *Journal of Pediatric Gastroenterology and Nutrition, 34*(2), 180-187

Boudry, G., Morise, A., Perrier, C., Seve, B., & Luron, I. (2006). L'allaitement artificiel modifie le développement post-natal de la barrière épithéliale intestinale et sa régulation nerveuse chez le porcelet de faible poids à la naissance. *Nutrition clinique et métabolisme, 20*, S78-S105

Boudry, G., Morise, A., Seve, B., & Le Huerou-Luron, I. (2011). Effect of Milk Formula Protein Content on Intestinal Barrier Function in a Porcine Model of LBW Neonates. *Pediatric Research, 69*(1), 4-9

Boudry, G., Péron, V., Lalles, J. P., & Sève, B. (2003). *Development of intestinal epithelial barrier function at weaning in piglets.* Paper presented at the 9th International Symposium on Digestive Physiology in Pigs, Canada. http://prodinra.inra.fr/record/69320

Boudry, G., Peron, V., Le Huerou-Luron, I., Lalles, J. P., & Seve, B. (2004). Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *Journal of Nutrition, 134*(9), 2256-2262

Boudry, G., Rome, V., Perrier, C., Jamin, A., Savary, G., & Le Huerou-Luron, I. (2014). A high-protein formula increases colonic peptide transporter 1 activity during neonatal life in low-birth-weight piglets and disturbs barrier function later in life. *British Journal of Nutrition, 112*(7), 1073-1080

Brigelius, R. (1985). Mixed disulfide: biological functions and increases. In H. Sies (Ed.), *Oxidative Stress* (pp. 243-272): Elsevier Science.

Brodsky, D., & Christou, H. (2004). Current concepts in intrauterine growth restriction. *Journal of Intensive Care Medicine, 19*(6), 307-319

Broer, S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews, 88*(1), 249-286

C

Carlberg, I., & Mannervik, B. (1985). Glutathione reductase. *Methods in Enzymology, 113*, 484-490

Chandel, N. S., Trzyna, W. C., McClintock, D. S., & Schumacker, P. T. (2000). Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *Journal of Immunology, 165*(2), 1013-1021

Chang, J., van der Hoeven, L., & Haddox, C. (1978). Glutathione reductase in the red blood cells. *Annals of Clinical and Laboratory Science, 8*(1), 23-29

Che, L., Xuan, Y., Hu, L., Liu, Y., Xu, Q., Fang, Z., Lin, Y., Xu, S., Wu, D., Zhang, K., & Chen, D. (2015). Effect of postnatal nutrition restriction on the oxidative status of neonates with intrauterine growth restriction in a pig model. *Neonatology, 107*(2), 93-99

Chelikani, P., Fita, I., & Loewen, P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences, 61*(2), 192-208

Chow, J. Y. C., Ma, L., & Cho, C. H. (1998). Involvement of Free Radicals and Histamine in the Potentiating Action of Cigarette Smoke Exposure on Ethanol-Induced Gastric Mucosal Damage in Rats. *Free Radical Biology and Medicine, 24*(7–8), 1285-1293

Chu, F. F., Esworthy, R. S., Ho, Y. S., Bermeister, M., Swiderek, K., & Elliott, R. W. (1997). Expression and chromosomal mapping of mouse Gpx2 gene encoding the gastrointestinal form of glutathione peroxidase, GPX-GI. *Biomedical and Environmental Sciences, 10*(2-3), 156-162

Circu, M. L., & Aw, T. Y. (2012). Intestinal redox biology and oxidative stress. *Seminars in Cell & Developmental Biology, 23*(7), 729-737

Clark, J. A., Doelle, S. M., Halpern, M. D., Saunders, T. A., Holubec, H., Dvorak, K., Boitano, S. A., & Dvorak, B. (2006). Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. *American Journal of Physiology Gastrointestinal and Liver Physiology, 291*(5), G938-949

Cooper, J. E. (1975). The use of the pig as an animal model to study problems associated with low birthweight. *Laboratory Animals, 9*(4), 329-336

D

D'Inca, R., Che, L., Thymann, T., Sangild, P. T., & Le Huërou-Luron, I. (2010a). Intrauterine growth restriction reduces intestinal structure and modifies the response to colostrum in preterm and term piglets. *Livestock Science, 133*(1-3), 20-22

D'Inca, R., Gras-Le Guen, C., Che, L., Sangild, P. T., & Le Huerou-Luron, I. (2011). Intrauterine growth restriction delays feeding-induced gut adaptation in term newborn pigs. *Neonatology, 99*(3), 208-216

D'Inca, R., Kloareg, M., Gras-Le Guen, C., & Le Huerou-Luron, I. (2010b). Intrauterine growth restriction modifies the developmental pattern of intestinal structure, transcriptomic profile, and bacterial colonization in neonatal pigs. *Journal of Nutrition, 140*(5), 925-931

Dammanahalli, K. J., Stevens, S., & Terkeltaub, R. (2012). Vanin-1 Pantetheinase Drives Smooth Muscle Cell Activation in Post-Arterial Injury Neointimal Hyperplasia. *PloS One, 7*(6)

Darmon, N., Pelissier, M. A., Heyman, M., Albrecht, R., & Desjeux, J. F. (1993). Oxidative Stress May Contribute to the Intestinal Dysfunction of Weanling Rats Fed a Low-Protein Diet. *Journal of Nutrition, 123*(6), 1068-1075

Davidge, S. T., Hubel, C. A., Brayden, R. D., Capeless, E. C., & Mclaughlin, M. K. (1992). Sera Antioxidant Activity in Uncomplicated and Preeclamptic Pregnancies. *Obstetrics and Gynecology, 79*(6), 897-901

Davies, K. J. (1995). Oxidative stress: the paradox of aerobic life. *Biochemical Society Symposia, 61*, 1-31

de Onis, M., Blossner, M., & Villar, J. (1998). Levels and patterns of intrauterine growth retardation in developing countries. *European Journal of Clinical Nutrition, 52 Suppl 1*, S5-15

De Quelen, F., Chevalier, J., Rolli-Derkinderen, M., Mourot, J., Neunlist, M., & Boudry, G. (2011). n–3 polyunsaturated fatty acids in the maternal diet modify the postnatal development of nervous regulation of intestinal permeability in piglets. *The Journal of Physiology, 589*(17), 4341-4352

De Vos, M., Huygelen, V., Van Raemdonck, G., Willemen, S., Fransen, E., Van Ostade, X., Casteleyn, C., Van Cruchten, S., & Van Ginneken, C. (2014). Supplementing formula-fed piglets with a low molecular weight fraction of bovine colostrum whey results in an improved intestinal barrier. *Journal of Animal Science, 92*(8), 3491-3501

Degroote, J., Michiels, J., Claeys, E., Ovyn, A., & De Smet, S. (2012). Changes in the pig small intestinal mucosal glutathione kinetics after weaning. *Journal of Animal Science, 90 Suppl 4*, 359-361

Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism, and Cardiovascular Diseases, 15*(4), 316-328

DeLeve, L. D., & Kaplowitz, N. (1991). Glutathione metabolism and its role in hepatotoxicity. *Pharmacology and Therapeutics, 52*(3), 287-305

Deneke, S. M., & Fanburg, B. L. (1989). Regulation of cellular glutathione. *American Journal of Physiology, 257*(4 Pt 1), L163-173

Deponte, M. (2013). Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochimica et Biophysica Acta, 1830*(5), 3217-3266

Dickinson, A. G., Hancock, J. L., Hovell, G. J. R., Taylor, C. S., & Wiener, G. (1962). The size of lambs at birth—a study involving egg transfer. *Animal Science, 4*(01), 64-79

Dicklin, M. E., Robinson, J. L., Lin, X., & Odle, J. (2006). Ontogeny and chain-length specificity of gastrointestinal lipases affect medium-chain triacylglycerol utilization by newborn pigs. *Journal of Animal Science, 84*(4), 818-825

Dixit, P., Jain, D. K., & Rajpoot, J. S. (2012). Differential effect of oxidative stress on intestinal apparent permeability of drugs transported by paracellular and transcellular route. *European Journal of Drug Metabolism and Pharmacokinetics, 37*(3), 203-209

Djavaheri-Mergny, M., Javelaud, D., Wietzerbin, J., & Besancon, F. (2004). NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells. *FEBS Letters, 578*(1-2), 111-115

Dobarganes, C., & Marquez-Ruiz, G. (2003). Oxidized fats in foods. *Current Opinion in Clinical Nutrition and Metabolic Care, 6*(2), 157-163

Drozdowski, L. A., Clandinin, T., & Thomson, A. B. (2010). Ontogeny, growth and development of the small intestine: Understanding pediatric gastroenterology. *World Journal of Gastroenterology, 16*(7), 787-799

Dunn, J. S. (1967). The fine structure of the absorptive epithelial cells of the developing small intestine of the rat. *Journal of Anatomy, 101*(Pt 1), 57-68

E

Erkens, T., Van Poucke, M., Vandesompele, J., Goossens, K., Van Zeveren, A., & Peelman, L. J. (2006). Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and longissimus dorsi muscle, and evaluation with PPARGC1A. *BMC Biotechnology, 6*, 41

Esworthy, R. S., Aranda, R., Martín, M. G., Doroshow, J. H., Binder, S. W., & Chu, F.-F. (2001). Mice with combined disruption of Gpx1 andGpx2 genes have colitis. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 281*(3), G848-G855

F

Fan, M. Z. (2003). Growth and ontogeny of the gastrointestinal tract. In R. J. Xu & P. D. Cranwell (Eds.), *Gastrointestinal Physiology and Nutrition of Neonatal Pigs.* (pp. 40-42). Leicestershire,U.K: Nottingham University Press.

Fanca-Berthon, P., Michel, C., Pagniez, A., Rival, M., van Seuningen, I., Darmaun, D., & Hoebler, C. (2009). Intrauterine Growth Restriction Alters Postnatal Colonic Barrier Maturation in Rats. *Pediatric Research, 66*(1), 47-52

Fasano, A. (2011). Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiological Reviews, 91*(1), 151-175

Ferenc, K., Pietrzak, P., Godlewski, M. M., Piwowarski, J., Kilianczyk, R., Guilloteau, P., & Zabielski, R. (2014). Intrauterine growth retarded piglet as a model for humans--studies on the perinatal development of the gut structure and function. *Reproductive Biology, 14*(1), 51-60

Ferraris, R. P. (2001). Dietary and developmental regulation of intestinal sugar transport. *Biochemical Journal, 360*(Pt 2), 265-276

Ferraris, R. P., & Diamond, J. (1997). Regulation of intestinal sugar transport. *Physiological Reviews, 77*(1), 257-302

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature, 408*(6809), 239-247

Flores, C., & McCord, J. M. (1997). Redox regulation by the HIV-1 Tat transcriptional factor. In J. G. Scandalios (Ed.), *Oxidative stress and the molecular biology of antioxidant defenses* (pp. 117-138). Plainview, N.Y.: Cold Spring Harbor Laboratory Press.

Foxcroft, G. B., G.; Dixon, W.; Hahn, M.; Harding, J.; Patterson, J.; Putman, T.; Sarmento, S.; Smit, M.; Tse WaiYue; Town, S. . (2007). *Consequences of selection for litter size on piglet development*. Paper presented at the Paradigms in pig science, Canada.

Franklin, C. C., Backos, D. S., Mohar, I., White, C. C., Forman, H. J., & Kavanagh, T. J. (2009). Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Molecular Aspects of Medicine, 30*(1-2), 86-98

Friel, J. K., Friesen, R. W., Harding, S. V., & Roberts, L. J. (2004). Evidence of Oxidative Stress in Full-Term Healthy Infants. *Pediatric Research, 56*(6), 878-882

Fuh, K. C., Meneshian, A., Patel, C. B., Takiar, V., & Bulkley, G. B. (2002). Signal transduction by reactive oxygen species: Alternative paradigms for signaling specificity. *Surgery, 131*(6), 601-612

Fung, C. M., White, J. R., Brown, A. S., Gong, H. Y., Weitkamp, J. H., Frey, M. R., & McElroy, S. J. (2016). Intrauterine Growth Restriction Alters Mouse Intestinal Architecture during Development. *PloS One, 11*(1)

G

Giri, D. K., & Aggarwal, B. B. (1998). Constitutive activation of NF-kappaB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. *Journal of Biological Chemistry, 273*(22), 14008-14014

Gitter, A. H., Wullstein, F., Fromm, M., & Schulzke, J. D. (2001). Epithelial barrier defects in ulcerative colitis: Characterization and quantification by electrophysiological imaging. *Gastroenterology, 121*(6), 1320-1328

Groschwitz, K. R., & Hogan, S. P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. *Journal of Allergy and Clinical Immunology, 124*(1), 3-20; quiz 21-22

Grotto, D., Santa Maria, L. D., Boeira, S., Valentini, J., Charao, M. F., Moro, A. M., Nascimento, P. C., Pomblum, V. J., & Garcia, S. C. (2007). Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. *Journal of Pharmaceutical and Biomedical Analysis, 43*(2), 619-624

Gu, X., Li, D., & She, R. (2002). Effect of weaning on small intestinal structure and function in the piglet. *Archiv für Tierernahrung, 56*(4), 275-286

Guilloteau, P., Zabielski, R., Hammon, H. M., & Metges, C. C. (2010). Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? *Nutrition Research Reviews, 23*(1), 4-22

Gundersen, H. J., & Jensen, E. B. (1987). The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy, 147*(Pt 3), 229-263

H

Hampson, D. J. (1986). Alterations in piglet small intestinal structure at weaning. *Research in Veterinary Science, 40*(1), 32-40

Han, D., Hanawa, N., Saberi, B., & Kaplowitz, N. (2006). Hydrogen peroxide and redox modulation sensitize primary mouse hepatocytes to TNF-induced apoptosis. *Free Radical Biology and Medicine, 41*(4), 627-639

Hancock, J. T., Desikan, R., & Neill, S. J. (2001). Role of reactive oxygen species in cell signalling pathways. *Biochemical Society Transactions, 29*(2), 345-349

Hansen, A. V., Strathe, A. B., Kebreab, E., France, J., & Theil, P. K. (2012). Predicting milk yield and composition in lactating sows: A Bayesian approach1. *Journal of Animal Science, 90*(7)

He, Q., Ren, P., Kong, X., Xu, W., Tang, H., Yin, Y., & Wang, Y. (2011). Intrauterine growth restriction alters the metabonome of the serum and jejunum in piglets. *Molecular Biosystems, 7*(7), 2147-2155

Heinonen, I., Rinne, P., Ruohonen, S. T., Ruohonen, S., Ahotupa, M., & Savontaus, E. (2014). The effects of equal caloric high fat and western diet on metabolic syndrome, oxidative stress and vascular endothelial function in mice. *Acta Physiologica (Oxford, England), 211*(3), 515-527

Heyman, M., Boudraa, G., Sarrut, S., Giraud, M., Evans, L., Touhami, M., & Desjeux, J. F. (1984). Macromolecular transport in jejunal mucosa of children with severe malnutrition: a quantitative study. *Journal of Pediatric Gastroenterology and Nutrition, 3*(3), 357-363

Heyman, M., Ducroc, R., Desjeux, J. F., & Morgat, J. L. (1982). Horseradish peroxidase transport across adult rabbit jejunum in vitro. *American Journal of Physiology, 242*(6), G558-564

Heyman, M., Grasset, E., Ducroc, R., & Desjeux, J. F. (1988). Antigen absorption by the jejunal epithelium of children with cow's milk allergy. *Pediatric Research, 24*(2), 197-202

Hu, C. H., Xiao, K., Luan, Z. S., & Song, J. (2013). Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. *Journal of Animal Science, 91*(3), 1094-1101

Huang, P., Feng, L., Oldham, E. A., Keating, M. J., & Plunkett, W. (2000). Superoxide dismutase as a target for the selective killing of cancer cells. *Nature, 407*(6802), 390-395

Huygelen, V., De Vos, M., Willemen, S., Fransen, E., Casteleyn, C., Van Cruchten, S., & Van Ginneken, C. (2014). Age-related differences in mucosal barrier function and morphology of the small intestine in low and normal birth weight piglets. *Journal of Animal Science, 92*(8), 3398-3406

Huygelen, V., De Vos, M., Willemen, S., Tambuyzer, B., Casteleyn, C., Knapen, D., Van Cruchten, S., & Van Ginneken, C. (2012). Increased intestinal barrier function in the small intestine of formula-fed neonatal piglets. *Journal of Animal Science, 90 Suppl 4*, 315-317

I

Iles, K. E., & Liu, R. M. (2005). Mechanisms of glutamate cysteine ligase (GCL) induction by 4-hydroxynonenal. *Free Radical Biology and Medicine, 38*(5), 547-556

Iwakiri, R., Rhoads, C. A., & Aw, T. Y. (1995). Determinants of hydroperoxide detoxification in diabetic rat intestine: effect of insulin and fasting on the glutathione redox cycle. *Metabolism: Clinical and Experimental, 44*(11), 1462-1468

J

Jacob, R. A., Aiello, G. M., Stephensen, C. B., Blumberg, J. B., Milbury, P. E., Wallock, L. M., & Ames, B. N. (2003). Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. *Journal of Nutrition, 133*(3), 740-743

Jolma, V. M., Kendall, K., & Koldovsky, O. (1980). Differences in the Development of Jejunum and Ileum as Observed in Fetal-Rat Intestinal Isografts - Possible Implications Related to the Villus Size Gradient. *American Journal of Anatomy, 158*(2), 211-215

Jones, R. M., Mercante, J. W., & Neish, A. S. (2012). Reactive oxygen production induced by the gut microbiota: pharmacotherapeutic implications. *Current Medicinal Chemistry, 19*(10), 1519-1529

Juarez, J. C., Manuia, M., Burnett, M. E., Betancourt, O., Boivin, B., Shaw, D. E., Tonks, N. K., Mazar, A. P., & Donate, F. (2008). Superoxide dismutase 1 (SOD1) is essential for H(2)O(2)-mediated oxidation and inactivation of phosphatases in growth factor signaling. *Proceedings of the National Academy of Sciences of the United States of America, 105*(20), 7147-7152

K

Kairisalo, M., Korhonen, L., Blomgren, K., & Lindholm, D. (2007). X-linked inhibitor of apoptosis protein increases mitochondrial antioxidants through NF-kappaB activation. *Biochemical and Biophysical Research Communications, 364*(1), 138-144

Kanazawa, K., & Ashida, H. (1998). Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism, 1393*(2-3), 349-361

Kaplowitz, N. (1981). The importance and regulation of hepatic glutathione. *Yale Journal of Biology and Medicine, 54*(6), 497-502

Keating, J., Bjarnason, I., Somasundaram, S., Macpherson, A., Francis, N., Price, A. B., Sharpstone, D., Smithson, J., Menzies, I. S., & Gazzard, B. G. (1995). Intestinal Absorptive-Capacity, Intestinal Permeability and Jejunal Histology in Hiv and Their Relation to Diarrhea. *Gut, 37*(5), 623-629

Kelly, D., & Coutts, A. G. P. (2000). Development of digestive and immunological function in neonates: role of early nutrition. *Livestock Production Science, 66*(2), 161-167

Kiliaan, A. J., Saunders, P. R., Bijlsma, P. B., Berin, M. C., Taminiau, J. A., Groot, J. A., & Perdue, M. H. (1998). Stress stimulates transepithelial macromolecular uptake in rat jejunum. *American Journal of Physiology, 275*(5 Pt 1), G1037-1044

Kim, J. C., Hansen, C. F., Mullan, B. P., & Pluske, J. R. (2012). Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Animal Feed Science and Technology, 173*(1-2), 3-16

Kim, S. W., Hurley, W. L., Hant, I. K., & Easter, R. A. (2000). Growth of nursing pigs related to the characteristics of nursed mammary glands. *Journal of Animal Science, 78*(5), 1313-1318

Kong, X., Thimmulappa, R., Craciun, F., Harvey, C., Singh, A., Kombairaju, P., Reddy, S. P., Remick, D., & Biswal, S. (2011). Enhancing Nrf2 pathway by disruption of Keap1 in myeloid leukocytes protects against sepsis. *American Journal of Respiratory and Critical Care Medicine, 184*(8), 928-938

Kotler, B. M., Kerstetter, J. E., & Insogna, K. L. (2013). Claudins, dietary milk proteins, and intestinal barrier regulation. *Nutrition Reviews, 71*(1), 60-65

Koutroubakis, I. E., Malliaraki, N., Dimoulios, P. D., Karmiris, K., Castanas, E., & Kouroumalis, E. A. (2004). Decreased Total and Corrected Antioxidant Capacity in Patients with Inflammatory Bowel Disease. *Digestive Diseases and Sciences, 49*(9), 1433-1437

Kowalski, D. P., Feeley, R. M., & Jones, D. P. (1990). Use of exogenous glutathione for metabolism of peroxidized methyl linoleate in rat small intestine. *Journal of Nutrition, 120*(9), 1115-1121

Kruidenier, L., Kuiper, I., van Duijn, W., Marklund, S. L., van Hogezand, R. A., Lamers, C. B., & Verspaget, H. W. (2003). Differential mucosal expression of three superoxide dismutase isoforms in inflammatory bowel disease. *Journal of Pathology, 201*(1), 7-16

Kurz, D. J., Decary, S., Hong, Y., Trivier, E., Akhmedov, A., & Erusalimsky, J. D. (2004). Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *Journal of Cell Science, 117*(Pt 11), 2417-2426

L

Lalles, J. P., Bosi, P., Smidt, H., & Stokes, C. R. (2007). Weaning - A challenge to gut physiologists. *Livestock Science, 108*(1-3), 82-93

Lalles, J. P., Boudry, G., Favier, C., Le Floc'h, N., Lurona, I., Montagne, L., Oswald, I. P., Pie, S., Piel, C., & Seve, B. (2004). Gut function and dysfunction in young pigs: physiology. *Animal Research, 53*(4), 301-316

Laukoetter, M. G., Bruewer, M., & Nusrat, A. (2006). Regulation of the intestinal epithelial barrier by the apical junctional complex. *Curr Opin Gastroenterol, 22*(2), 85-89

Lavrovsky, Y., Schwartzman, M. L., Levere, R. D., Kappas, A., & Abraham, N. G. (1994). Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proceedings of the National Academy of Sciences of the United States of America, 91*(13), 5987-5991

Le Dividich, J., Herpin, P., Paul, E., & Strullu, F. (1997). Effect of fat content of colostrum on voluntary colostrum intake and fat utilization in newborn pigs. *Journal of Animal Science, 75*(3)

Le Dividich, J., Rooke, J. A., & Herpin, P. (2005). Nutritional and immunological importance of colostrum for the new-born pig. *Journal of Agricultural Science, 143*, 469-485

Le Dividich, J., & Seve, B. (2000). Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet. *Domestic Animal Endocrinology, 19*(2), 63-74

Liu, Z., Wang, S., & Hu, M. (2009). Chapter 11 - Oral Absorption Basics: Pathways, Physico-chemical and Biological Factors Affecting Absorption *Developing Solid Oral Dosage Forms* (pp. 263-288). San Diego: Academic Press.

Lu, L., Bao, Y., Khan, A., Goldstein, A. M., Newburg, D. S., Quaroni, A., Brown, D., & Walker, W. A. (2008). Hydrocortisone Modulates Cholera Toxin Endocytosis by Regulating Immature Enterocyte Plasma Membrane Phospholipids. *Gastroenterology, 135*(1), 185-193.e181

Lu, S. C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB Journal, 13*(10), 1169-1183

Lu, S. C. (2009). Regulation of glutathione synthesis. *Molecular Aspects of Medicine, 30*(1-2), 42-59

Lu, S. C. (2013). Glutathione synthesis. *Biochimica Et Biophysica Acta-General Subjects, 1830*(5), 3143-3153

Lupertz, R., Chovolou, Y., Kampkotter, A., Watjen, W., & Kahl, R. (2008). Catalase overexpression impairs TNF-alpha induced NF-kappaB activation and sensitizes MCF-7 cells against TNF-alpha. *Journal of Cellular Biochemistry, 103*(5), 1497-1511

Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A., & Said, H. M. (2004). TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *American Journal of Physiology Gastrointestinal and Liver Physiology, 286*(3), G367-376

Maeda, T., Miyazono, Y., Ito, K., Hamada, K., Sekine, S., & Horie, T. (2010). Oxidative stress and enhanced paracellular permeability in the small intestine of methotrexate-treated rats. *Cancer Chemotherapy and Pharmacology, 65*(6), 1117-1123

Magee, T. R., Han, G., Cherian, B., Khorram, O., Ross, M. G., & Desai, M. (2008). Downregulation of Transcription Factor Peroxisome Proliferator-activated Receptor in Programmed Hepatic Lipid Dysregulation and Inflammation in Intrauterine Growth Restricted Offspring. *American Journal of Obstetrics and Gynecology, 199*(3), 271.e271-271.e275

Martin, F., Penet, M. F., Malergue, F., Lepidi, H., Dessein, A., Galland, F., de Reggi, M., Naquet, P., & Gharib, B. (2004). Vanin-1(-/-) mice show decreased NSAID- and Schistosoma-induced intestinal inflammation associated with higher glutathione stores. *Journal of Clinical Investigation, 113*(4), 591-597

Martin, M. G., Wu, S. V., & Walsh, J. H. (1993). Hormonal control of intestinal Fc receptor gene expression and immunoglobulin transport in suckling rats. *Journal of Clinical Investigation, 91*(6), 2844-2849

Martin, M. G., Wu, S. V., & Walsh, J. H. (1997). Ontogenetic development and distribution of antibody transport and Fc receptor mRNA expression in rat intestine. *Digestive Diseases and Sciences, 42*(5), 1062-1069

McCracken, B. A., Spurlock, M. E., Roos, M. A., Zuckermann, F. A., & Gaskins, H. R. (1999). Weaning anorexia may contribute to local inflammation in the piglet small intestine. *Journal of Nutrition, 129*(3), 613-619

McIntire, D. D., Bloom, S. L., Casey, B. M., & Leveno, K. J. (1999). Birth weight in relation to morbidity and mortality among newborn infants. *New England Journal of Medicine, 340*(16), 1234-1238

McKie, A. T., Zammit, P. S., & Naftalin, R. J. (1999). Comparison of cattle and sheep colonic permeabilities to horseradish peroxidase and hamster scrapie prion protein in vitro. *Gut, 45*(6), 879-888

McMillen, I. C., & Robinson, J. S. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological Reviews, 85*(2), 571-633

Ménard, S., Cerf-Bensussan, N., & Heyman, M. (2010). Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunology, 3*(3), 247-259

Menon, R. (2014). Oxidative Stress Damage as a Detrimental Factor in Preterm Birth Pathology. *Frontiers in Immunology, 5*, 567

Meyer, M., Schreck, R., & Baeuerle, P. A. (1993). H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO Journal, 12*(5), 2005-2015

Michiels, J., De Vos, M., Missotten, J., Ovyn, A., De Smet, S., & Van Ginneken, C. (2013). Maturation of digestive function is retarded and plasma antioxidant capacity lowered in fully weaned low birth weight piglets. *British Journal of Nutrition, 109*(1), 65-75

Mickiewicz, M., Zabielski, R., Grenier, B., Le Normand, L., Savary, G., Holst, J. J., Oswald, I. P., Metges, C. C., & Guilloteau, P. (2012). Structural and functional development of small intestine in intrauterine growth retarded porcine offspring born to gilts fed diets with differing protein ratios throughout pregnancy. *Journal of Physiology and Pharmacology, 63*(3), 225-239

Moeser, A. J., Borst, L. B., Overman, B. L., & Pittman, J. S. (2012). Defects in small intestinal epithelial barrier function and morphology associated with peri-weaning failure to thrive syndrome (PFTS) in swine. *Research in Veterinary Science, 93*(2), 975-982

Moeser, A. J., Klok, C. V., Ryan, K. A., Wooten, J. G., Little, D., Cook, V. L., & Blikslager, A. T. (2007a). Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *American Journal of Physiology Gastrointestinal and Liver Physiology, 292*(1), G173-181

Moeser, A. J., Ryan, K. A., Nighot, P. K., & Blikslager, A. T. (2007b). Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *American Journal of Physiology Gastrointestinal and Liver Physiology, 293*(2), G413-421

Mohr, D., Umeda, Y., Redgrave, T. G., & Stocker, R. (1999). Antioxidant defenses in rat intestine and mesenteric lymph. *Redox Report, 4*(3), 79-87

Montagne, L., Boudry, G., Favier, C., Le Huerou-Luron, I., Lalles, J. P., & Seve, B. (2007). Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *British Journal of Nutrition, 97*(1), 45-57

Morgan, B., Sobotta, M. C., & Dick, T. P. (2011). Measuring E(GSH) and H2O2 with roGFP2-based redox probes. *Free Radical Biology and Medicine, 51*(11), 1943-1951

Morgan, M. J., & Liu, Z.-g. G. (2011). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research, 21*(1), 103-115

Mowat, A. M., & Agace, W. W. (2014). Regional specialization within the intestinal immune system. *Nature Reviews Immunology, 14*(10), 667-685

Murphy, M. P. (2012). Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. *Antioxidants & Redox Signaling, 16*(6), 476-495

Mutlu-Turkoglu, U., Ademoglu, E., Ibrahimoglu, L., Aykac-Toker, G., & Uysal, M. (1998). Imbalance between lipid peroxidation and antioxidant status in preeclampsia. *Gynecologic and Obstetric Investigation, 46*(1), 37-40

N

Nabuurs, M. J. A., Hoogendoorn, A., Vandermolen, E. J., & Vanosta, A. L. M. (1993). Villus Height and Crypt Depth in Weaned and Unweaned Pigs, Reared under Various Circumstances in the Netherlands. *Research in Veterinary Science, 55*(1), 78-84

Nenoi, M., Ichimura, S., Mita, K., Yukawa, O., & Cartwright, I. L. (2001). Regulation of the Catalase Gene Promoter by Sp1, CCAAT-recognizing Factors, and a WT1/Egr-related Factor in Hydrogen Peroxide-resistant HP100 Cells. *Cancer Research, 61*(15), 5885-5894

Nielsen, F., Mikkelsen, B. B., Nielsen, J. B., Andersen, H. R., & Grandjean, P. (1997). Plasma malondialdehyde as biomarker for oxidative stress: Reference interval and effects of life-style factors. *Clinical Chemistry, 43*(7), 1209-1214

Nunn, A. V. W., Bell, J., & Barter, P. (2007). The integration of lipid-sensing and anti-inflammatory effects: how the PPARs play a role in metabolic balance. *Nuclear Receptor, 5*, 1-1

O

Oates, P. S., & West, A. R. (2006). Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. *World Journal of Gastroenterology, 12*(27), 4281-4295

Otterbein, L. E., & Choi, A. M. K. (2000). Heme oxygenase: colors of defense against cellular stress. *American Journal of Physiology - Lung Cellular and Molecular Physiology, 279*(6), L1029-L1037

Overman, E. L., Rivier, J. E., & Moeser, A. J. (2012). CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. *PloS One, 7*(6), e39935

P

Pacha, J. (2000). Development of intestinal transport function in mammals. *Physiological Reviews, 80*(4), 1633-1667

Pappenheimer, J. R., & Reiss, K. Z. (1987). Contribution of Solvent Drag through Intercellular-Junctions to Absorption of Nutrients by the Small-Intestine of the Rat. *Journal of Membrane Biology, 100*(2), 123-136

Perry, J. S., & Rowell, J. G. (1969). Variations in Foetal Weight and Vascular Supply Along Uterine Horn of Pig. *Journal of Reproduction and Fertility, 19*(3), 527-&

Pettigrew, J. E., Cornelius, S. G., Moser, R. L., Heeg, T. R., Hanke, H. E., Miller, K. P., & Hagen, C. D. (1986). Effects of oral doses of corn oil and other factors on preweaning survival and growth of piglets. *Journal of Animal Science, 62*(3), 601-612

Pie, S., Lalles, J. P., Blazy, F., Laffitte, J., Seve, B., & Oswald, I. P. (2004). Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *Journal of Nutrition, 134*(3), 641-647

Pluske, J. R., Hampson, D. J., & Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science, 51*(1-3), 215-236

Pluske, J. R., Le Dividich, J., & Verstegen, M. W. A. (2003). *Weaning the pig* (J. L. D. J.R. Pluske, and M.W.A. Verstegen Ed.): Wageningen Academic Publishers

Podolsky, D. K. (1999). Mucosal Immunity and Inflammation V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 277*(3), G495-G499

Puchal, A. A., & Buddington, R. K. (1992). Postnatal-Development of Monosaccharide Transport in Pig Intestine. *American Journal of Physiology, 262*(5), G895-G902

Quesnel, H., Farmer, C., & Devillers, N. (2012). Colostrum intake: Influence on piglet performance and factors of variation. *Livestock Science, 146*(2–3), 105-114

Quiniou, N., Dagorn, J., & Gaudre, D. (2002). Variation of piglets birth weight and consequences on subsequent performance. *Livestock Production Science, 78*(1), 63-70

Rao, R. K., Basuroy, S., Rao, V. U., Karnaky, K. J., & Gupta, A. (2002). Tyrosine phosphorylation and dissociation of occludin-Z0-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *Biochemical Journal, 368*, 471-481

Rath, P. C., & Aggarwal, B. B. (2001). Antiproliferative effects of IFN-alpha correlate with the downregulation of nuclear factor-kappa B in human Burkitt lymphoma Daudi cells. *Journal of Interferon and Cytokine Research, 21*(7), 523-528

Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology, 27*(3), 199-217

Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., & Potter, D. W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Analytical Biochemistry, 106*(1), 55-62

Reinhart, G. A., Simmen, F. A., Mahan, D. C., White, M. E., & Roehrig, K. L. (1992). Intestinal development and fatty acid binding protein activity of newborn pigs fed colostrum or milk. *Biology of the Neonate, 62*(2-3), 155-163

Remels, A. H., Langen, R. C., Gosker, H. R., Russell, A. P., Spaapen, F., Voncken, J. W., Schrauwen, P., & Schols, A. M. (2009). PPARgamma inhibits NF-kappaB-dependent transcriptional activation in skeletal muscle. *American Journal of Physiology Endocrinology and Metabolism, 297*(1), E174-183

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology & Medicine, 49*(11), 1603-1616

Riccio, P., & Rossano, R. (2015). Nutrition facts in multiple sclerosis. *ASN Neuro, 7*(1)

Robles, R., Palomino, N., & Robles, A. (2001). Oxidative stress in the neonate. *Early Human Development, 65 Suppl*, S75-81

Rojo, A. I., Salinas, M., Martin, D., Perona, R., & Cuadrado, A. (2004). Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB. *Journal of Neuroscience, 24*(33), 7324-7334

Rueda-Clausen, C. F., Dolinsky, V. W., Morton, J. S., Proctor, S. D., Dyck, J. R., & Davidge, S. T. (2011). Hypoxia-induced intrauterine growth restriction increases the susceptibility of rats to high-fat diet-induced metabolic syndrome. *Diabetes, 60*(2), 507-516

S

Sanderson, I. R., & Walker, W. A. (1993). Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology, 104*(2), 622-639

Sangild, P. T., Schmidt, M., Elnif, J., Bjornvad, C. R., Westrom, B. R., & Buddington, R. K. (2002). Prenatal development of gastrointestinal function in the pig and the effects of fetal esophageal obstruction. *Pediatric Research, 52*(3), 416-424

Sankaran, S., & Kyle, P. M. (2009). Aetiology and Pathogenesis of IUGR. *Best Practice & Research in Clinical Obstetrics & Gynaecology, 23*(6), 765-777

Saunders, P. R., Kosecka, U., McKay, D. M., & Perdue, M. H. (1994). Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *American Journal of Physiology, 267*(5 Pt 1), G794-799

Schieber, M., & Chandel, N. S. (2014). ROS Function in Redox Signaling and Oxidative Stress. *Current Biology, 24*(10), R453-R462

Schreiber, J., Jenner, R. G., Murray, H. L., Gerber, G. K., Gifford, D. K., & Young, R. A. (2006). Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America, 103*(15), 5899-5904

Schutze, S., Wiegmann, K., Machleidt, T., & Kronke, M. (1995). TNF-induced activation of NF-kappa B. *Immunobiology, 193*(2-4), 193-203

Shan, X. Q., Aw, T. Y., & Jones, D. P. (1990). Glutathione-dependent protection against oxidative injury. *Pharmacology and Therapeutics, 47*(1), 61-71

Sharpe, H. B. (1966). Pre-weaning mortality in a herd of large white pigs. *British Veterinary Journal, 122*(3), 99-111

Shen, Y., Wan, H., Zhu, J., Fang, Z., Che, L., Xu, S., Lin, Y., Li, J., & Wu, D. (2015). Fish Oil and Olive Oil Supplementation in Late Pregnancy and Lactation Differentially Affect Oxidative Stress and Inflammation in Sows and Piglets. *Lipids, 50*(7), 647-658

Sheth, P., Basuroy, S., Li, C. Y., Naren, A. P., & Rao, R. K. (2003). Role of phosphatidylinositol 3-kinase in oxidative stress-induced disruption of tight junctions. *Journal of Biological Chemistry, 278*(49), 49239-49245

Skrzypek, T., Piedra, J. L. V., Skrzypek, H., Kazimierczak, W., Szymanczyk, S., Pawlowska, M., & Zabielski, R. (2007a). Intestinal villi structure during the development of pig and wild boar crossbreed neonates. *Livestock Science, 109*(1-3), 38-41

Skrzypek, T., Piedra, J. L. V., Skrzypek, H., Kazimierczak, W., Szymanczyk, S. E., & Zabielski, R. (2010). Changes in pig small intestinal absorptive area during the first 14 days of life. *Livestock Science, 133*(1-3), 53-56

Skrzypek, T., Valverde Piedra, J. L., Skrzypek, H., Kazimierczak, W., Biernat, M., & Zabielski, R. (2007b). Gradual disappearance of vacuolated enterocytes in the small intestine of neonatal piglets. *Journal of Physiology and Pharmacology, 58 Suppl 3*, 87-95

Smith, F., Clark, J. E., Overman, B. L., Tozel, C. C., Huang, J. H., Rivier, J. E., Blikslager, A. T., & Moeser, A. J. (2010). Early weaning stress impairs development of mucosal barrier function in the porcine intestine. *American Journal of Physiology Gastrointestinal and Liver Physiology, 298*(3), G352-363

Snoeck, V., Goddeeris, B., & Cox, E. (2005). The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes Infect, 7*(7-8), 997-1004

Söderholm, J. D., Streutker, C., Yang, P. C. C., Paterson, C., Singh, P. K., McKay, D. M., Sherman, P. M., Croitoru, K., & Perdue, M. H. (2004). Increased epithelial uptake of protein antigens in the ileum of Crohn's disease mediated by tumour necrosis factor alpha. *Gut, 53*(12), 1817-1824

Sporn, M. B., & Liby, K. T. (2012). NRF2 and cancer: the good, the bad and the importance of context. *Nature Reviews Cancer, 12*(8), 564-571

Spreeuwenberg, M. A., Verdonk, J. M., Gaskins, H. R., & Verstegen, M. W. (2001). Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. *Journal of Nutrition, 131*(5), 1520-1527

Surh, Y. J., Kundu, J. K., Na, H. K., & Lee, J. S. (2005). Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *Journal of Nutrition, 135*(12), 2993s-3001s

Swanson, A. M., & David, A. L. (2015). Animal models of fetal growth restriction: Considerations for translational medicine. *Placenta, 36*(6), 623-630

T

Tauler Riera, P. (2012). Redox Status. In F. C. Mooren (Ed.), *Encyclopedia of Exercise Medicine in Health and Disease* (pp. 751-753). Berlin, Heidelberg: Springer Berlin Heidelberg.

Telemo, E., Westrom, B. R., Ekstrom, G., & Karlsson, B. W. (1987). Intestinal macromolecular transmission in the young rat: influence of protease inhibitors during development. *Biology of the Neonate, 52*(3), 141-148

Tenhunen, R., Marver, H. S., & Schmid, R. (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proceedings of the National Academy of Sciences of the United States of America, 61*(2), 748-755

Thomson, A., Hemphill, D., & Jeejeebhoy, K. N. (1998). Oxidative stress and antioxidants in intestinal disease. *Digestive Diseases, 16*(3), 152-158

Trahair, J. F., & Sangild, P. T. (2002). Chapter 1 Studying the development of the small intestine: philosophical and anatomical perspectives. In P. C. G. B. W. R. Zabielski & E. Salek (Eds.), *Biology of Growing Animals* (Vol. Volume 1, pp. 1-54): Elsevier.

Tsang, C. K., Liu, Y., Thomas, J., Zhang, Y. J., & Zheng, X. F. S. (2014). Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat Commun, 5*

Tsukita, S., Furuse, M., & Itoh, M. (2001). Multifunctional strands in tight junctions. *Nature Reviews: Molecular Cell Biology, 2*(4), 285-293

Tsunada, S., Iwakiri, R., Noda, T., Fujimoto, K., Fuseler, J., Rhoads, C. A., & Aw, T. Y. (2003). Chronic exposure to subtoxic levels of peroxidized lipids suppresses mucosal cell turnover in rat small intestine and reversal by glutathione. *Digestive Diseases and Sciences, 48*(1), 210-222

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Reviews: Immunology, 9*(11), 799-809

U

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research, 35*(Web Server issue), W71-74

V

Van Der Hulst, R. R. W. J., Von Meyenfeldt, M. F., Van Kreel, B. K., Thunnissen, F. B. J. M., Brummer, R.-J. M., Arends, J.-W., & Soeters, P. B. (1998). Gut Permeability, Intestinal Morphology, and Nutritional Depletion. *Nutrition, 14*(1), 1-6

van Elburg, R. M., Fetter, W. P. F., Bunkers, C. M., & Heymans, H. S. A. (2003). Intestinal permeability in relation to birth weight and gestational and postnatal age. *Archives of Disease in Childhood, 88*(1), 52-55

Vandervliet, A., & Bast, A. (1992). Role of Reactive Oxygen Species in Intestinal-Diseases. *Free Radical Biology and Medicine, 12*(6), 499-513

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*(7), RESEARCH0034

Varady, J., Eder, K., & Ringseis, R. (2011). Dietary oxidized fat activates the oxidative stress-responsive transcription factors NF-kappaB and Nrf2 in intestinal mucosa of mice. *European Journal of Nutrition, 50*(8), 601-609

Varady, J., Ringseis, R., & Eder, K. (2012). Dietary moderately oxidized oil induces expression of fibroblast growth factor 21 in the liver of pigs. *Lipids in Health and Disease, 11*, 34

Vergauwen, H., Tambuyzer, B., Jennes, K., Degroote, J., Wang, W., De Smet, S., Michiels, J., & Van Ginneken, C. (2015). Trolox and Ascorbic Acid Reduce Direct and Indirect Oxidative Stress in the IPEC-J2 Cells, an In Vitro Model for the Porcine Gastrointestinal Tract. *PloS One, 10*(3), e0120485

Vile, G. F., Basu-Modak, S., Waltner, C., & Tyrrell, R. M. (1994). Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America, 91*(7), 2607-2610

Vlessis, A. A., & Mela-Riker, L. (1989). Perinatal development of heart, kidney, and liver mitochondrial antioxidant defense. *Pediatric Research, 26*(3), 220-226

W

Walker, W. A. (1979). Gastrointestinal host defence: importance of gut closure in control of macromolecular transport. *Ciba Foundation Symposium*(70), 201-219

Walter, J. K., Rueckert, C., Voss, M., Mueller, S. L., Piontek, J., Gast, K., & Blasig, I. E. (2009). The oligomerization of the coiled coil-domain of occludin is redox sensitive. *Annals of the New York Academy of Sciences, 1165*, 19-27

Wang, J., Chen, L., Li, D., Yin, Y., Wang, X., Li, P., Dangott, L. J., Hu, W., & Wu, G. (2008a). Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *Journal of Nutrition, 138*(1), 60-66

Wang, J. J., Chen, L. X., Li, P., Li, X. L., Zhou, H. J., Wang, F. L., Li, D. F., Yin, Y. L., & Wu, G. Y. (2008b). Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. *Journal of Nutrition, 138*(6), 1025-1032

Wang, T., Huo, Y. J., Shi, F., Xu, R. J., & Hutz, R. J. (2005). Effects of intrauterine growth retardation on development of the gastrointestinal tract in neonatal pigs. *Biology of the Neonate, 88*(1), 66-72

Wang, W., Degroote, J., Van Ginneken, C., Van Poucke, M., Vergauwen, H., Dam, T. M., Vanrompay, D., Peelman, L. J., De Smet, S., & Michiels, J. (2016). Intrauterine growth restriction in neonatal piglets affects small intestinal mucosal permeability and mRNA expression of redox-sensitive genes. *FASEB Journal, 30*(2), 863-873

Wang, X., Lin, G., Liu, C., Feng, C., Zhou, H., Wang, T., Li, D., Wu, G., & Wang, J. (2014). Temporal proteomic analysis reveals defects in small-intestinal development of porcine fetuses with intrauterine growth restriction. *Journal of Nutritional Biochemistry, 25*(7), 785-795

Wang, X., Wu, W., Lin, G., Li, D., Wu, G., & Wang, J. (2010). Temporal proteomic analysis reveals continuous impairment of intestinal development in neonatal piglets with intrauterine growth restriction. *Journal of Proteome Research, 9*(2), 924-935

Weldy, C. S., Luttrell, I. P., White, C. C., Morgan-Stevenson, V., Cox, D. P., Carosino, C. M., Larson, T. V., Stewart, J. A., Kaufman, J. D., Kim, F., Chitaley, K., & Kavanagh, T. J. (2013). Glutathione (GSH) and the GSH synthesis gene Gclm modulate plasma redox and vascular responses to acute diesel exhaust inhalation in mice. *Inhalation Toxicology, 25*(8), 444-454

Wendel, A., & Feuerstein, S. (1980). Role of Glutathione-Peroxidase in P-450-Mediated Drug-Induced Lipid-Peroxidation. *Journal of the American Oil Chemists Society, 57*(2), A145-A146

West, A. P., Shadel, G. S., & Ghosh, S. (2011). Mitochondria in innate immune responses. *Nature Reviews: Immunology, 11*(6), 389-402

Westrom, B. R., Svendsen, J., Ohlsson, B. G., Tagesson, C., & Karlsson, B. W. (1984). Intestinal transmission of macromolecules (BSA and FITC-labelled dextrans) in the neonatal pig. Influence of age of piglet and molecular weight of markers. *Biology of the Neonate, 46*(1), 20-26

White, C. C., Viernes, H., Krejsa, C. M., Botta, D., & Kavanagh, T. J. (2003). Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. *Analytical Biochemistry, 318*(2), 175-180

Wijtten, P. J., van der Meulen, J., & Verstegen, M. W. (2011). Intestinal barrier function and absorption in pigs after weaning: a review. *British Journal of Nutrition, 105*(7), 967-981

Wilson, J. M., & Casanova, J. E. (2000). Development of Endocytosis in the Intestinal Epithelium. In I. R. Sanderson & W. A. Walker (Eds.), *Development of the Gastrointestinal Tract* (pp. 71-81): B.C. Decker.

Winters, L. M., Cummings, J. N., & Stewart, H. A. (1947). A study of factors affecting survival from birth to weaning and total weaning weight of the litter in swine. *Journal of Animal Science, 6*(3), 288-296

Wiyaporn, M., Thongsong, B., & Kalandakanond-Thongsong, S. (2013). Growth and small intestine histomorphology of low and normal birth weight piglets during the early suckling period. *Livestock Science, 158*(1-3), 215-222

Wootton, R., Flecknell, P. A., Royston, J. P., & John, M. (1983). Intrauterine Growth-Retardation Detected in Several Species by Non-Normal Birth-Weight Distributions. *Journal of Reproduction and Fertility, 69*(2), 659-663

Wu, G., Bazer, F. W., Burghardt, R. C., Johnson, G. A., Kim, S. W., Li, X. L., Satterfield, M. C., & Spencer, T. E. (2010a). Impacts of amino acid nutrition on pregnancy outcome in pigs: Mechanisms and implications for swine production. *Journal of Animal Science, 88*, E195-E204

Wu, G., Bazer, F. W., Cudd, T. A., Meininger, C. J., & Spencer, T. E. (2004a). Maternal nutrition and fetal development. *Journal of Nutrition, 134*(9), 2169-2172

Wu, G., Bazer, F. W., Datta, S., Johnson, G. A., Li, P., Satterfield, M. C., & Spencer, T. E. (2008). Proline metabolism in the conceptus: implications for fetal growth and development. *Amino Acids, 35*(4), 691-702

Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Board-invited review: intrauterine growth retardation: implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., & Turner, N. D. (2004b). Glutathione metabolism and its implications for health. *Journal of Nutrition, 134*(3), 489-492

Wu, W. Z., Wang, X. Q., Wu, G. Y., Kim, S. W., Chen, F., & Wang, J. J. (2010b). Differential composition of proteomes in sow colostrum and milk from anterior and posterior mammary glands1. *Journal of Animal Science, 88*(8), 2657-2664

X

Xia, C., Hu, J., Ketterer, B., & Taylor, J. B. (1996). The organization of the human GSTP1-1 gene promoter and its response to retinoic acid and cellular redox status. *Biochemical Journal, 313 (Pt 1)*, 155-161

Xu, H., Collins, J. F., & Ghishan, F. K. (2012). Chapter 14 - Molecular Physiology of Gastrointestinal Function during Development A2 - Wood, Leonard R. JohnsonFayez K. GhishanJonathan D. KaunitzJuanita L. MerchantHamid M. SaidJackie D *Physiology of the Gastrointestinal Tract (Fifth Edition)* (pp. 415-449). Boston: Academic Press.

Xu, R. J. (1996). Development of the newborn GI tract and its relation to colostrum milk intake: A review. *Reproduction Fertility and Development, 8*(1), 35-48

Xu, R. J., Mellor, D. J., Birtles, M. J., Reynolds, G. W., & Simpson, H. V. (1994). Impact of Intrauterine Growth-Retardation on the Gastrointestinal-Tract and the Pancreas in Newborn Pigs. *Journal of Pediatric Gastroenterology and Nutrition, 18*(2), 231-240

Xu, R. J., Mellor, D. J., Tungthanathanich, P., Birtles, M. J., Reynolds, G. W., & Simpson, H. V. (1992). Growth and Morphological-Changes in the Small and the Large-Intestine in Piglets during the 1st 3 Days after Birth. *Journal of Developmental Physiology, 18*(4), 161-172

Xu, R. J., Wang, F., & Zhang, S. H. (2000). Postnatal adaptation of the gastrointestinal tract in neonatal pigs: a possible role of milk-borne growth factors. *Livestock Production Science, 66*(2), 95-107

Y

Yagi, K. (1998). Simple assay for the level of total lipid peroxides in serum or plasma. *Methods in Molecular Biology, 108*, 101-106

Yang, Y., Dieter, M. Z., Chen, Y., Shertzer, H. G., Nebert, D. W., & Dalton, T. P. (2002). Initial characterization of the glutamate-cysteine ligase modifier subunit Gclm(-/-) knockout mouse. Novel model system for a severely compromised oxidative stress response. *Journal of Biological Chemistry, 277*(51), 49446-49452

Ye, D., Ma, I., & Ma, T. Y. (2006). Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. *American Journal of Physiology Gastrointestinal and Liver Physiology, 290*(3), G496-504

Ye, J. (2008). Regulation of PPARgamma function by TNF-alpha. *Biochemical and Biophysical Research Communications, 374*(3), 405-408

Yin, J., Ren, W., Liu, G., Duan, J., Yang, G., Wu, L., Li, T., & Yin, Y. (2013). Birth oxidative stress and the development of an antioxidant system in newborn piglets. *Free Radical Research, 47*(12), 1027-1035

Yin, J., Wu, M. M., Xiao, H., Ren, W. K., Duan, J. L., Yang, G., Li, T. J., & Yin, Y. L. (2014). Development of an antioxidant system after early weaning in piglets. *Journal of Animal Science, 92*(2), 612-619

Yuan, T. L., Zhu, Y. H., Shi, M., Li, T. T., Li, N., Wu, G. Y., Bazer, F. W., Zang, J. J., Wang, F. L., & Wang, J. J. (2015). Within-litter variation in birth weight: impact of nutritional status in the sow. *Journal of Zhejiang University-Science B, 16*(6), 417-435

Z

Zabielski, R., Godlewski, M. M., & Guilloteau, P. (2008). Control of Development of Gastrointestinal System in Neonates. *Journal of Physiology and Pharmacology, 59*, 35-54

Zabielski, R., Le Huerou-Luron, I., & Guilloteau, P. (1999). Development of gastrointestinal and pancreatic functions in mammalians (mainly bovine and porcine species): influence of age and ingested food. *Reproduction Nutrition Development, 39*(1), 5-26

Zhang, B., Lo, C., Shen, L., Sood, R., Jones, C., Cusmano-Ozog, K., Park-Snyder, S., Wong, W., Jeng, M., Cowan, T., Engleman, E. G., & Zehnder, J. L. (2011). The role of vanin-1 and oxidative stress-related pathways in distinguishing acute and chronic pediatric ITP. *Blood, 117*(17), 4569-4579

Zhang, H., Chen, Y., Li, Y., Yang, L., Wang, J., & Wang, T. (2014). Medium-chain TAG attenuate hepatic oxidative damage in intra-uterine growth-retarded weanling piglets by improving the metabolic efficiency of the glutathione redox cycle. *British Journal of Nutrition, 112*(6), 876-885

Zhang, H., Court, N., & Forman, H. J. (2007). Submicromolar concentrations of 4-hydroxynonenal induce glutamate cysteine ligase expression in HBE1 cells. *Redox Report, 12*(1), 101-106

Zhang, H., Li, Y., & Wang, T. (2015). Antioxidant capacity and concentration of redox-active trace mineral in fully weaned intra-uterine growth retardation piglets. *Journal of Animal Science and Biotechnology, 6*, 48

Zhang, H. Z., Malo, C., & Buddington, R. K. (1997). Suckling induces rapid intestinal growth and changes in brush border digestive functions of newborn pigs. *Journal of Nutrition, 127*(3), 418-426

Zhu, L. H., Zhao, K. L., Chen, X. L., & Xu, J. X. (2012). Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. *Journal of Animal Science, 90*(8), 2581-2589

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research, 31*(13), 3406-3415

CURRICULUM VITAE

Wei Wang 王蔚

Tel: +32(0)468 22 01 65 E-mail: wei.wang@ugent.be

EDUCATION

❖ **Ghent University(UGENT); Gent, Belgium** DEC. 2011 – JUN.2016

Ph.D. Applied Biological Science, Swine Nutrition

Directed by Prof. Stefaan De Smet, Dr. Joris Michiels and Prof. Chris Van Ginneken

❖ **Sichuan Agricultural University(SICAU); Sichuan, China** 2008 - 2011

Master's Degree of Agriculture, Swine Nutrition

Directed by Prof. Daiwen Chen

❖ **Southwest University of Science and Technology (SWUST); Sichuan, China** 2004 - 2008

Bachelor's degree of Agriculture, Animal Science

GRANTS AND AWARDS

❖ **Special Research Funding for Chinese Ph.D. Students (Ghent University)** MAR. 2014 - DEC. 2015

❖ **Scholarship from Chinese Scholarship Council** DEC. 2011 - DEC. 2015

❖ **THE OUTSTANDING GRADUATE of Sichuan Province, P.R. China** 2008

❖ **National Scholarship by Ministry of Education P.R. China** 2007
(only top 1% of Chinese undergraduates could be awarded)

A1 PUBLICATIONS

Wang, W., Degroote, J., Van Ginneken, C., Van Poucke, M., Vergauwen, H., Dam, T. M., Vanrompay, D., Peelman, L. J., De Smet, S., & Michiels, J. (2016). Intrauterine growth restriction in neonatal piglets affects small intestinal mucosal permeability and mRNA expression of redox-sensitive genes. FASEB Journal, 30(2), 863-873. (IF 2015=5.299. Ranking: Biology 7/86)

Wang, W., Chen, H., Yu, B., Mao, X., & Chen, D. (2014). Tissue deposition and residue depletion of melamine in fattening pigs following oral administration. Food Additives & Contaminants. Part A: Chemistry,

Analysis, Control, Exposure & Risk Assessment, 31(1), 7-14 (IF 2015=1.878. Ranking: Chemistry applied, 27/71).

Chen, H., **Wang, W.**, Degroote, J., Possemiers, S., Chen, D. W., De Smet, S., & Michiels, J. (2015). Arabinoxylan in Wheat Is More Responsible Than Cellulose for Promoting Intestinal Barrier Function in Weaned Male Piglets. Journal of Nutrition, 145(1), 51-58 (IF2015 =3.740, Ranking: Nutrition&Diatetics, 17/78).

Vergauwen, H., Tambuyzer, B., Jennes, K., Degroote, J., **Wang, W.**, De Smet, S., Michiels, J., & Van Ginneken, C. (2015). Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an in vitro model for the porcine gastrointestinal tract. PloS One, 10(3), e0120485 (IF2015 =3.057, Ranking: Multidisciplinary Sciences, 11/63).

ACKNOWLEDGEMENT

The PhD study is a journey full with challenges. It took me four and a half years to arrive the destination. The journey is such a difficult one, so it is impossible to finish it without a lot of people's help.

Firstly, I would like to express my deepest appreciations to Prof. Stefaan De Smet. Thank you for offering me the entrance ticket to this adventure. As a promoter, you have taken great responsibility to make sure my every step was towards the right direction. Besides the guidance in academic field, you also showed me how to be a responsible person. I really appreciate all the support you have offered me, especially during the difficult moments. Without your guidance and support, I do not think I can finish the PhD. To me, you are the one and only best professor. **Dr. Joris Michiels and Prof. Chris Van Ginneken,** my dear copromoters, thank you for all the patient and careful guidance on my experiments, my manuscripts and my thesis. Joris, thank you for involving me in such a good project, for the selfless share of your knowledge and the careful control of the quality of my work. Chris, I appreciate it very much your extra working hours for reviewing my manuscripts and thesis, especially during your holidays. Both of you have made tremendous contribution to my PhD study, I hope it is a PhD you expected.

Many thanks to my jury members; Prof. Mieke Uyttendaele, Prof. Walter Gerrits, Prof. Katleen Raes, Prof. Richard Ducatelle, and Prof. Lianqiang Che, Thank you for your professional suggestions of my thesis. Most importantly, I appreciate it very much that all of you have taken a kind and smooth way to help me to improve my work. I cannot do other things to repay your kindness instead of trying my best to improve this thesis, hope this final version is what you want to see.

I would like to thank the nice people in LAB MONOGASTRIC, where I worked for four years. **Jeroen**, one more time, my deepest thank for everything you have done for me during the last four years. I am so lucky to work together with you on the same project, and I am 100% sure you will have a great PhD, because the professional research background and rich practical experience are perfectly combined on you. **Ann** and **Tessa,** thank you for all the support you have offered me during my stay. Ann, thank you for your comfort every time when I am down. Tessa, we have the same lab coats, this means a lot to me. I am really proud that I can be the member of LAB MONOGASTRIC.

Daisy, Annick, Sabine, Erik and Els, I respect you all very much, and the help you have offered me since the very beginning, they are priceless for me. I want to let you know that I hope one day I can give some return to all the things you have done for me. Please remember if there is anything I can do, I would like to do it, always.

Prof. Veerle Fieves, although you are not my promotor, it is very nice that every time when we met in the kitchen, you would ask me about my progress and give some encouragement. Thank you for that, for me it means you treat me almost as your own student. I know you are doing great job leading the ruminant group, and I heard from your student that you are a great professor, is it nice to hear this comment? ☺

The main lab analysis in this thesis was done in Vakgroep Voeding, Genetica en Ethologie (LDG), leading by **Prof. Luc Peelman,** and Laboratory for Immunology and Animal Biotechnology, leading by **Prof. Daisy Vanrompay**. I would first thank the nice professors I have met in this two labs, they have given me great trust and access to all the equipment necessary for my analysis, and they have provided me a lot of support and care of my manuscript. Thank you! Mario, Sara, Linda, Ruben, Dominique and Jozefin, thank you for all the kindness to me. Tho, thank you for your professional and patient guidance of western blotting, I wish you very good luck in your research. Annelien, thanks for arranging me the practical stuffs.

I have to say I am very lucky that I have entered a lab with super international atmosphere. As colleagues, we can discuss methods, statistical analysis, normal data, strange data, paper… After work, we are friends, we share our good and bad life experience, we laugh at each other's stupid moment, we cheer each other on at difficult moment, and we stand up for some strange things happened, no matter what is right or wrong. These names will be in my memory forever, they are: Thomas & Mariel (the doctoral couple), Sasitorn (Pu, my Thai BFF), Lin, Umaporn (Toony), Alexis, Nympha and Claudio. Thank you for your kindness and friendship, I will treasure all the things you have given to me forever. Oh, Thomas, I think I owe you several sandwiches for translating my summary to Dutch? ☺ Luckily I have you as an example to check the practical things I should do for my defense. Thank you!

My thanks also go to the lovely colleagues in LANUPRO. Alberto, Alex, Bruno, Cecilia, Charlotte, Elisa, Frederik, Géoffrey, Joris Missotons, Joaquin, Lore, Marc, Malar, Marlene, Marta, Maryam,

Michele, Matteo, Mornica, Nico, Noémie, Norma, Paiwan, Pieter, Pooya, Sandra, Sieglinde, Sophie, Sylvia, Thomas (a musician working on insects), Stefaan, Stijn, Quan, Rahman, Thanh, Wantanee (Jeab), and Zaneta. I wish you all the very best!

I have met also great local (actually to me you are foreigners… ☺) and foreign (non-Belgian) people. Olivier, it was really nice to meet you, thanks for all the greetings and helps you have offered to me! My French running coach, Florent Forest, my thank is not only for your "mean" training, but also for your cakes, for your driving when I am tired, although I think your driving is not that better than mine…☺ Edwin, thank you for looking for the farm for the Chinese delegation to visit, you have made something "impossible" being possible!

As a foreigner here in Belgium, a country has a very different culture from China, I never feel lonely because the great staff in Education section in China Embassy in Belgium. I want to thank our ex-counselor of education, Luxin Wang, current counselor of education, Hongjian Tao, ex-executive director of education section, Changwei Ma, and the current executive director of education section, Yantao Su and Kai Ma, for all the supports during the last four years. Of course I cannot forget all the support from my lovely Chinese friends. Longhui, I promised that I will write a sentence about your offer of home-made lunch during my writing, a lot of energy! Honglin, Linghong (I put your name together intentionally to let them think who is who… ☺), Ping, Ziyu, thank you all for the accompany in the lab. I hope you can have a lot of success; no matter you will continue doing research or finding a job in private section in the future. My dear friends, Kunpeng, Lijie, Qian, Wenjuan, Nan, Wenwen & Ou, Xingzhe & Riccard, Anni & Yang, Xiaochen & Junzhi, Limin&Bairen, Jiajia & Bing, Jun & Yufeng, Lei&Xing, Yuechao&Qi. It was great to meet you all here. Most of you already have a happy family, keep on being happy as such!

My special thanks goes to **Prof. Daiwen Chen** in Sichuan Agricultural University, my supervisor of my master thesis. The 3 years study in his team has given me a solid foundation for my laster PhD study. Another person I cannot forget to give my appreciations is **Prof. Junjun Wang** in China Agricultural University. Thank you for all the guidance to my research, my future career choice and all the help you have offered during my visit in China last year. You are a great professor, maybe not as great as my promoter yet, but I am sure you will be in the near future! ☺

My dear sister, Qing Wang and my brother in law, Bo Zou, thank you for taking care Papa and Mama. I know quite clear that I can be totally concentrate on my work, because there is nothing I have to

worried about with my family. Qing, you are the great sister, ever! I know how much you have done for our family and for me, I hope that you know that no matter where I am, I am always with you. Yinuo Zou, thank you for all the happiness you have brought to our family. I am quite sure that you will be able to understand these words soon. My only niece, I love you.

Last but most important, I want to thank my parents. 女儿没有辜负你们的期望，拿到了博士学位。四年多来，与我承受的压力相比，你们为我的担心和煎熬只多不少。我只希望在今后的日子里能用我拥有的一切回报你们。爸爸妈妈，再一次深深的感谢你们！我爱你们。

Wei Wang 王蔚

22 June 2016